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ENVIRONMENTAL DNA DETECTION AS AN EFFECTIVE TOOL FOR DELIMITING SPATIAL DISTRIBUTION OF RARE AND INVASIVE SPECIES, AND ASSESSING COMMUNITY IN FLOWING, FRESHWATER ECOSYSTEMS

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by

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Environmental DNA Detection as an Effective Tool for Delimiting Spatial Distribution of Rare and Invasive Species, and Assessing Community in Flowing, Freshwater Ecosystems

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DECLARATION OF CO-AUTHORSHIP AND PREVIOUS PUBLICATION

I. Co-Authorship Declaration

I hereby declare that this thesis incorporates material that is result of joint research, as follows: This thesis also incorporates the outcome of a joint research undertaken in collaboration with Dr. Ryan P. Walter under the supervision of my supervisor Dr. Daniel D. Heath and co-supervisor Dr. Nicholas E. Mandrak; this collaboration covers Chapter 2 and Chapter 3. In all cases, the key ideas, primary contributions, experimental designs, data analysis and interpretation, were performed by the author (myself), with additional input on data analysis, interpretation of data, and written discussion by co-authors.

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II. **Declaration of Previous Publications**

This thesis includes material from two original papers that have been submitted for publication in peer reviewed journals, as follows:

Thesis Chapter	Publication Title	Publication Status
Chapter 2	Balasingham KD, Walter RP, Heath DD. "Residual eDNA detection sensitivity assessed by quantitative real-time PCR in a river ecosystem"	Submitted to *Molecular Ecology Resources*
Chapter 3	Balasingham KD, Walter RP, Mandrak NE, Heath DD. "Environmental DNA detection of invasive and rare fish species with their associated freshwater fish community in two Great Lakes tributaries"	Submitted to *Molecular Ecology*

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ABSTRACT

Environmental DNA (eDNA) can be extracted from water samples to determine target species presence and location, important for the detection of at-risk species or early invaders. I determined if eDNA can be used to identify the presence and location of target species. I quantified the signal strength of residual eDNA in a flowing system, while the target eDNA source entered the system at a fixed site (i.e. source site). I found that the strongest signals were always at the source site indicating that this method can be used to locate low-abundance species in rivers. I also found that eDNA and next-generation sequencing (NGS) detected 51 of 67 fishes (76.1%) from two large tributaries. Detections included three target species at risk and one target invasive species which contributed to 77.0% of the NGS data, indicating that eDNA and NGS can be used to monitor native communities in highly invaded habitats.

DEDICATION

I dedicate this thesis to my family and Stephen B. Chau
for their continued support, optimism, and love over the years.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

LIST OF TABLES

CHAPTER 3

LIST OF ABBREVIATIONS

BLAST(n): Basic Local Alignment Search Tool (nucleotide)

BOLD: Barcode of Life Database

bp: Base Pairs

BSA: Bovine Serum Albumin

CI: Confidence Interval

COI: Cytochrome *c* Oxidase Subunit I

CPUE: Catch Per Unit Effort

Ct: Cycle Threshold

CTAB: Cetyl Trimethylammonium Bromide

cyt*b*: Cytochrome *b*

ddH_2O: Double Distilled Water

D-loop: Displacement Loop

dNTP: Deoxynucleotide Triphosphate

eDNA: Environmental Deoxyribonucleic Acid

FREC: Freshwater Restoration Ecology Centre

LOD/LOQ: Limit of Detection/Quantification

$MgCl_2$: Magnesium Chloride

NGS: Next-Generation Sequencing

PS1: Community Primer Set 1

qRT-PCR: Quantitative Real-Time Polymerase Chain Reaction

RNase A: Ribonuclease A

rRNA: Ribosomal Ribonucleic Acid

SEM: Standard Error of the Mean

Taq: *Thermus aquaticus*

TE Buffer: Tris-EDTA (Ethylenediaminetetraacetic Acid)

UV-B: Ultraviolet B

Chapter 1

GENERAL INTRODUCTION

Covering more than 70% of the Earth's surface and containing over 90% of the habitable area, marine and freshwater ecosystems are among the most diverse systems globally (Geist 2011; Appeltans et al. 2012). Only 3% of all surface water is freshwater, and out of that small portion, roughly 1.5% is accessible for consumption while also sustaining approximately 125 000 freshwater species (9.5% of all known animal species) in lakes, ponds and rivers (Shiklomanov 1993; Dudgeon et al. 2015). Freshwater habitats are biodiversity "hotspots," that are areas containing a large number of species, many of which are considered to be at-risk (Mächler et al. 2014). Being a vital resource that not only supplies domestic and commercial water needs, and a variety of aquatic species for human consumption, humans have developed large settlements around freshwater systems (Dudgeon et al. 2006; Strayer & Dudgeon 2010), ultimately leading to the degradation of water quality and aquatic life. Habitat fragmentation due to dams, pollution from agriculture and wastewater treatment plants, and the introduction of exotic species have all resulted in a serious decrease in global freshwater biodiversity (Mandrak & Cudmore 2010). The greatest diversity of freshwater species in Canada resides in the Great Lakes-St. Lawrence basin (hereafter the Great Lakes basin), which is also the world's largest freshwater ecosystem. It has over 100 fish species established throughout the lakes and associated tributaries (Roth et al. 2013), while comprising 20% of accessible surface freshwater (Cudmore-Vokey & Crossman 2000). Hence, it is not surprising that approximately 63% of Canada's total population and about 24% of the total US population is settled around the Great Lakes basin, resulting in high urbanization and industrialization in the area (Chu et al. 2015; Campbell et al. 2015). Over 180 exotic species have been introduced and established in the Great Lakes within the past century, having been brought over by ballast water from transport

vessels and through the live trade, stocking, and manmade canals (Ricciardi 2006; Sylvester & MacIsaac 2010). As a result, conservation efforts in the Great Lakes region require large-scale, repeated surveys in an attempt to detect the early spread of known harmful species, identify newly introduced species and monitor species at risk to prevent further loss of local biodiversity.

Conventional Sampling Methods

Researchers and decision-makers are faced with difficult choices when trying to determine which species need to be monitored or managed for conservation purposes. Unfortunately, critically endangered species will go extinct because there simply is not enough time or resources to monitor or manage all critical species (Hager et al. 2006). The key is prevention through preservation of remaining local biodiversity, which can be effectively done by focusing management efforts on high-priority species. These species often include bioindicators species which reflect the quality of the environment (Holt & Miller 2010), species with highly detrimental impacts (e.g. invasive species), or species at risk. Once a target species is selected, sampling and monitoring of the species over time is implemented to gather important information such as population density and distribution, habitat use, resource use, spatial movement, and life history patterns. However, in water ecosystems, surveying aquatic species poses several challenges. First and foremost, humans cannot readily move or see as well underwater as on land, and we rely on technology for monitoring purposes. This is especially difficult for species that inhabit difficult to reach areas, are cryptic, elusive, or very small (Spear et al. 2015). Also, evaluating a species' state may require its capture for blood samples or physiological measurements, requiring the use of nets, seines, electrofishing or other invasive capture techniques to collect specimens (Cooke et al. 2013). These methods can be expensive, strenuous, lengthy, and cause unintentional harm to targeted and non-targeted species in the

area, and the habitat (Santas et al. 2013; Mächler et al. 2014; Evans et al. 2016); contrary to conservation management. Recently, the introduction - or rather, the integration – of molecular techniques into conservation research has improved methods for studying endangered and invasive species by incorporating microsatellite analysis, next-generation sequencing (NGS), gene expression, genetic diet analysis, and within the scope of this thesis, non-invasive DNA sampling using environmental DNA (DeSalle & Amato 2004; Santas et al. 2013; Hennessy 2015).

Environmental DNA

Environmental DNA (eDNA) analysis is a non-invasive surveying approach that uses sampled water to infer the presence or absence of aquatic species, instead of physically capturing specimens. The water contains DNA molecules in tissues released into the surrounding environment via blood, urine, shedding skin cells, eggs and sperm, or fecal matter from all organisms inhabiting the environment (Taberlet et al. 2012). Water samples can be processed and eDNA extracted for use with molecular genetic marker loci and a suite of molecular genetic detection methodologies. The data generated can then be used for target species detection in habitats associated with the water samples, leading to the identification of potential biodiversity hotspots or areas vulnerable to invasion.

Persistence of eDNA in aquatic environments is dependent upon several abiotic factors such as temperature, UV-B exposure, microbial metabolism, dilution, and water chemistry (Takahara et al. 2012; Turner et al. 2015; Strickler et al. 2015), and biotic factors such as species biomass and population density, diet, body size, and life stage (Klymus et al. 2015; Spear et al. 2015). Despite several factors working against the longevity of eDNA molecules, eDNA analysis has proven to be successful in several recent studies focusing on a variety of aquatic species that includes amphibians (Ficetola et al. 2008; Pilliod et al. 2014; Fukumoto et al. 2015),

invertebrates (Goldberg et al. 2013; Deiner & Altermatt 2014; Mächler et al. 2014), and fishes (Thomsen et al. 2012; Jerde et al. 2011; Sigsgaard et al. 2015; Port et al. 2016). For example, shed eDNA degrades quickly making it an ideal tool for the detection of *recent* or *nearby* species (Barnes et al. 2014); an important factor when determining the distribution for species of conservation concern (e.g., species at risk or invasive). Likewise, not all eDNA molecules will be subjected to equal degradation and may be retained in the environment as a molecular signature of species present in the system recently, or in high abundance (Ficetola et al. 2008). As eDNA approaches use species identification from water samples instead of physical capture, it is imperative to employ several controls throughout the entire process to minimize type I errors (false positives) and type II errors (false negatives).

Type I and Type II Errors

False positives and false negatives are a concern with eDNA analysis and several protocols have been developed to minimize those risks. False positives occur when a target species has been detected in a sample but it is not present in the study system, whereas, false negatives occur when a target species is present in the system but there was no detectable target eDNA in the sample (Rees et al. 2014). False positives occur primarily due to contamination of samples between sites or in the lab, but this source of error can be avoided or greatly reduced by sterilizing all equipment and including blank samples during field sampling, water filtering, eDNA extraction, and PCR amplification (Bohmann et al. 2014; Thomsen & Willerslev 2015). False negatives can occur in samples that are highly inhibited for DNA extraction or amplification due to the presence of humic or fulvic acids (Matheson et al. 2010; McKee et al. 2015), or other DNA modifying substances. Inhibitory effects can be minimized with the use of appropriate DNA extraction kits (Amberg et al. 2015), use of sample replicates during

PCR, and well-designed molecular markers with high PCR efficiency (Kwok et al. 1990; Bohmann et al. 2014). In addition, the majority of eDNA movement (displacement) is downstream in lotic systems, resulting in species detections downstream from their actual location (Deiner & Altermatt 2014; Jane et al. 2015; Pilliod et al. 2014). This effect can be corrected for by collecting samples at various upstream sites and at multiple time points (Bohmann et al. 2014). However, the issue of identifying the source location for eDNA detection is still problematic in flowing ecosystems.

Genetic Markers

Although eDNA analysis currently cannot describe the physical characteristics of the individual (i.e. biomass, sex, age), there are tools such as quantitative real-time polymerase chain reaction (qRT-PCR) that can provide relative estimates of target species density (Thomsen et al. 2012; Goldberg et al. 2013; Pilliod et al. 2013; Klymus et al. 2015). Amplification of DNA traditionally uses end-point PCR, followed by visualization of the resulting PCR products on agarose gels for confirmation of presence through detection of appropriate-sized bands; however, this is less sensitive than qRT-PCR (Turner et al. 2014; Wilcox et al. 2013). qRT-PCR detects the fluorescence of amplified target DNA at each cycle of the polymerase chain reaction, and when the fluorescence reaches a threshold value (at the linear amplification stage), the cycle threshold (Ct) value is identified (Tuomi et al. 2010; Nathan et al. 2014). As an example, samples containing high quantities of target DNA will produce more fluorescence earlier generating a lower Ct value, as it requires fewer cycles to exceed the threshold fluorescence value.

The detection of the target species eDNA relies on efficient molecular genetic markers that can be species-specific or generic (i.e. targets a broad range of species). Species-specific

primers are designed primarily in conserved gene regions with little variation in sequence within the target species, but with greater sequence variation amongst species (Wilcox et al. 2013). The goal of species-specific primers it to isolate one or a few critical species (i.e. species at risk or invasive) in eDNA samples (MacDonald et al. 2014; Miya et al. 2015). However, since water samples collected from natural environments will contain a mixture of DNA from all organisms that co-occur with the target species, targeting several species in the community can be useful for large-scale community analysis. For community analysis, generic primers are designed to target multiple species in one PCR. Generic primers also target conserved regions; however, due to differences in sequences between species even in conserved regions, generic primer sets often incorporate degenerate nucleotide sites. Degenerate nucleotides are positions in the sequence that have more than one possible base (Linhart & Shamir 2002). Both types of primers can be designed targeting one or multiple highly conserved genes, such as 12S rRNA, cytochrome *b* oxidase (cyt*b*), or cytochrome *c* oxidase subunit I (COI). These genes are ideal targets because they do not have high rates of evolution in conserved regions and are, thus, conserved within and between species (Leray et al. 2013), but also have highly polymorphic regions that can be used to distinguish species. For many species, the ideal target gene is COI as it can distinguish species that are closely related (Hebert et al. 2003; Meusnier et al. 2008) and there are large, available databases such as the Barcode of Life Database (BOLD) and Genbank that contain thousands of COI sequences available for more than 5000 species (Ward et al. 2009).

Next-Generation Sequencing

The emergence of new sequencing technologies such as next-generation sequencing (NGS) platforms have allowed eDNA analysis to target several species at once, which is

beneficial for community analysis (Rees et al. 2014). NGS can process millions of sequences simultaneously, and the generated sequence reads can be matched against sequences from available databases (e.g. Genbank) to verify taxonomy (Shokralla et al. 2012; Taberlet et al. 2012). The high processing capability via PCR-NGS eDNA analysis has also proven to be useful for the early detection of invasive species (Ficetola et al. 2008; Jerde et al. 2011; Goldberg et al. 2013; Tréguier et al. 2014; Piaggio et al. 2014; Simmons et al. 2015), and the detection of rare species (Janosik & Johnston 2015; Laramie et al. 2015; Sigsgaard et al. 2015). Often, eDNA studies have focused on targeting one or a few species and used traditional Sanger sequencing to verify taxonomy of amplified products (Taberlet et al. 2012). However, improvements to NGS platforms and decreasing costs allow NGS platforms to be more available and an ideal sequencing approach for eDNA samples (Rees et al. 2014).

Thesis Objectives

This thesis focuses on eDNA analysis as a tool for the detection of target species in flowing (river) systems. I use eDNA persistence in aquatic environments to evaluate signal strength of residual eDNA in a flowing, uncontrolled system, after the removal of an eDNA source (Chapter 2). This thesis also examines eDNA analysis as an effective tool for the detection of multiple target species, including species at risk and invasive species in two large Great Lakes tributaries located in southern Ontario (Chapter 3). Chapter 3 also introduces the potential for fish community analysis using the same eDNA approach. Throughout this study, eDNA is analyzed with quantitative and semi-quantitative processing platforms such as qRT-PCR and PCR-NGS, as both provide sensitive and specific detection of eDNA signals. The use of qRT-PCR and PCR-NGS for eDNA analysis will likely replace traditional end-point PCR for most ecological and conservation applications.

In Chapter 2, I use two sampling regimes in Little River: (1) beginning at the source site (where eDNA enters the river at a fixed point) and moving downstream, and (2) beginning downstream and moving upstream towards the source site. Water movement affects eDNA concentration and degradation (Dejean et al. 2011), which can influence the eDNA detection sensitivity of a target species. This is especially important in cases where the target species has left the system but residual eDNA may remain in the system for a short amount of time, indicating its recent presence (Barnes et al. 2014). Downstream migration of eDNA is a critical factor in flowing systems as it precludes the determination of species location, unless repeated sampling coupled with quantitative target eDNA detection is employed. To address samples with low eDNA concentrations, I used qRT-PCR to assign Ct values (up to a maximum of 55 qRT-PCR cycles) to calculate the overall mean Ct of samples per site. Sites with low mean Ct values have higher eDNA concentrations in the samples, representing samples that were likely collected when less time had passed (less dilution) and/or closer to the source site.

In Chapter 3, I use a hybrid primer PCR approach with eDNA and NGS to assess the distribution and biodiversity of three species at risk (Eastern Sand Darter *Ammocrypta pellucida*, Silver Shiner *Notropis photogenis*, Northern Madtom *Noturus stigmosus*) and one invasive species (Round Goby *Neogobius melanostomus*) in two large Great Lakes tributaries; Sydenham River and Grand River. Species primers (designed to amplify target and closely related species), together with a generic, fish community primer (designed to target 63 additional species known to reside in the two sampled rivers) were used to amplify a region of the COI gene from extracted eDNA, and the resulting multiplexed PCR was sequenced via NGS. The subsequent presence/absence data was used to test for significant species co-occurrences. In Chapter 3, I also evaluate the overall effectiveness of eDNA detection by comparing its species detection rates against detections obtained from conventional capture-based methods. Spatial

distribution inferences based on presence/absence of target species is important, however, species relationships (e.g. significant species co-occurrences) may also offer important insight as to why target species occur where they do, and which native species may be essential for effective species management.

These chapters focus on eDNA genetics as an important tool for conservation of endangered species and management of invasive species that are issues of high concern throughout the Great Lakes basin. I show that eDNA can be quantitative and used to identify the proximate location and recent presence of a recently removed target species using eDNA signal strength. I demonstrate that eDNA analysis is an alternative surveying method that can be used in dynamic, uncontrolled flowing aquatic systems. I argue that targeting certain species using eDNA analysis should incorporate targeting known native species to increase confidence in the eDNA protocol.

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CHAPTER 2

RESIDUAL EDNA DETECTION SENSITIVITY ASSESSED BY QUANTITATIVE REAL-TIME PCR IN A RIVER ECOSYSTEM[1]

Introduction

Detecting aquatic species without using conventional capture methods such as netting, or electrofishing (which are often harmful to the target individual and their ecosystem) is of particular importance when monitoring the distribution of rare species (Jones 1992; Baldwin et al. 1996; Port et al. 2006). In aquatic habitats, species DNA is retained in the environment from biological sources (e.g. shed skin cells, urine, feces, sperm, eggs, mucous). This environmental DNA (eDNA) offers an alternative approach to surveying presence of the species of interest through their genetic information accessed by sampling their DNA in the water, rather than physically capturing individuals (Ficetola et al. 2008; Goldberg et al. 2011; Taberlet et al. 2012; Wilson & Wright 2013). The integration of molecular genetic techniques and aquatic ecology (eDNA analysis) has resulted in greater detection sensitivity for rare species and early detection of aquatic invasive species (Goldberg et al. 2013; Thomsen et al. 2012; Jerde et al. 2012). Since eDNA analysis uses DNA sequences of the target species for detection, it has reduced errors associated with taxonomic identification, making it ideal for species that are taxonomically cryptic, present at low abundance, or inhabit inaccessible areas (Jerde et al. 2011; Dejean et al. 2012; Pilliod et al. 2013). Furthermore, the ease of obtaining water samples relative to obtaining live specimens, greatly decreases field time and costs and reduces the risk of harming individuals or their environment.

Several recent studies have illustrated eDNA analysis as a powerful tool in the detection of rare aquatic species, including both at-risk and early invaders. For example, studies have

[1] Balasingham KD, Walter RP, Heath DD. Residual eDNA detection sensitivity assessed by quantitative real-time PCR in a river ecosystem. Submitted to *Molecular Ecology Resources*.

successfully detected invasive species such as the American Bullfrog (*Lithobates catesbeianus*) in southwestern France wetlands (Ficetola et al. 2008), Asian carps (*Hypophthalmichthys* spp.) in the Great Lakes basin (Jerde et al. 2011; Jerde et al. 2012), Red Swamp Crayfish (*Procambarus clarkii*) in ponds in northwestern France (Tréguier et al. 2014), Burmese Python (*Python bivittatus*) in Florida ponds (Piaggio et al. 2014), Bighead Carp (*Hypophthalmichthys nobilis*) in the Muskingum River Watershed in Ohio (Simmons et al. 2015) and New Zealand Mudsnail (*Potamopyrgus antipodarum*) invading freshwater systems globally (Goldberg et al. 2013). Studies have also been successful in detecting species at risk, such as Chinook Salmon (*Oncorhynchus tshawytscha*) in the Upper Columbia River (Laramie et al. 2015) and the European Weather Loach (*Misgurnus fossilis*) in Denmark (Sigsgaard et al. 2015). These studies employ species-specific polymerase chain reaction (PCR) primers that can be designed to target highly conserved mitochondrial or nuclear regions for the detection of the target species. The PCR products are then visualized on agarose gels to identify the presence of the target band, inferring the presence of a species for a particular eDNA sample. Positive PCR products are then sequenced for confirmation of the target species detection (Ficetola et al. 2008; Jerde et al. 2011; Thomsen & Willerslev 2014).

However, traditional end-point PCR is not quantitative and while it can be used to infer species presence, it does not provide information on signal strength, nor any quantitative estimate of detection sensitivity. Quantitative real-time polymerase chain reaction (qRT-PCR) can provide relative abundance estimation, given some assumptions (Thomsen et al. 2012; Goldberg et al. 2013; Pilliod et al. 2013; Klymus et al. 2015). Additionally, qRT-PCR is more sensitive for detecting low concentrations of template DNA, which is often a limiting factor for aquatic eDNA (Wilcox et al. 2013). Quantifying template eDNA concentrations has been identified as an important component of eDNA analysis, as an improvement over simple

presence data, and more studies incorporating qRT-qPCR for eDNA detection analyses are being published (Takahara et al. 2012; Pilliod et al. 2013; Wilcox et al. 2013; Uchii et al. 2016). For example, Uchii et al. (2016) used qRT-PCR to quantify eDNA detections from non-native genotypes of Common Carp (*Cyprinus carpio*) in a controlled system, and found a positive correlation between calculated template eDNA concentrations and the known biomass of the target species. Similarly, Thomsen et al. (2011) measured the concentration of template eDNA via qRT-PCR analysis and reported a positive correlation between eDNA concentration and amphibian abundance. eDNA template concentrations are influenced by target species abundance and biomass coupled with the rate of DNA degradation influenced by temperature, microbial metabolism, and chemical/physical breakdown (Barnes & Turner 2016). The relationship between the concentration of eDNA and target species abundance becomes more complex in flowing environments such as rivers, as the movement of eDNA molecules adds to DNA concentration dilution and degradation. Nonetheless, qRT-PCR applications for eDNA analysis may be suitable for mapping eDNA concentrations in flowing systems to pinpoint locations of the target species based on quantified template DNA concentration gradients.

We define residual eDNA as the eDNA molecules that persist in the environment after removal of the source. While eDNA has been observed to degrade exponentially with time (Barnes et al. 2014), controlled laboratory experiments have shown that some eDNA molecules may persist longer than expected. Several studies reported eDNA retention times ranging from 7 to 25 days after the removal of the target freshwater species (Dejean et al. 2011; Thomsen et al. 2012; Goldberg et al. 2013; Barnes et al. 2014; Pilliod et al. 2014; Piaggio et al. 2014; Merkes et al. 2014; Strickler et al. 2015), however these studies used artificial and controlled environments.

The goal of this project is to quantify the detectability of residual eDNA from surface water samples in a natural flowing ecosystem two hours after the removal of the eDNA source. Water that held juvenile Atlantic Salmon (*Salmo salar*) was used as the eDNA source for this study to determine: (1) how far downstream can residual Atlantic Salmon eDNA be detected from a fixed eDNA source site within a short period of time after source water was exhausted and, (2) the signal strength of the residual eDNA relative to distance from the source. To accomplish this, we sampled our study river at uniform distances downstream from the eDNA source after stopping the inflow of source DNA water, and measured eDNA detection signal strengths using qRT-PCR. While a few studies have quantified the persistence of eDNA after removal of a target species in controlled environments, to our knowledge none have yet examined the interactions between the movement and retention of eDNA molecules in natural flowing ecosystems, after the eDNA source (i.e. species) is removed. Furthermore, our use of qRT-PCR for eDNA detection allows a quantitative analysis of the spatial gradient of residual eDNA concentration. We predicted that Atlantic Salmon eDNA concentrations would decrease at greater downstream distances, as dilution and degradation associated with the flow of eDNA molecules reduces retention. Such a functional relationship between spatial distance and eDNA signal would allow for the determination of point sources of eDNA, and possibly, a relative estimate of target species abundance.

Materials & Methods

Study Area & Study Species

The study river, Little River, is located in southwestern Ontario and is approximately 65 km^2 in surface area, 12 km in length, and flows into Lake St. Clair (Fig. 2.1). Little River was chosen as it does not contain the experimental target species, Atlantic Salmon, and was easily

accessible for sampling. Also, approximately 400 L of Atlantic Salmon water was readily available for use as a source of eDNA. The water came from a recirculation reservoir that fed rearing tanks holding 102 juvenile Atlantic Salmon (approximately 40 g each) and is located at the University of Windsor Freshwater Restoration Ecology Centre (FREC, LaSalle, ON, Canada).

Water Sampling

2014 Experiment: Two barrels containing the Atlantic Salmon water, each holding approximately 200 L, were set up on land next to Little River (42°16'56.1" N, 82°54'45.6" W; hereafter referred to as the source site; Fig. 2.1). This site was chosen due to easy access and setup of the barrels on an elevated area, required for gravity flow. The water was released into the river at a discharge rate of approximately 2.3 x 10^{-6} $m^3 \cdot s^{-1}$; the river flow was approximately 0.12 $m^3 \cdot s^{-1}$. Atlantic Salmon water flow started at 15:00 (Aug. 22) and stopped when the water supply was exhausted at approximately 09:00 on August 24 (total flow time: ~42 hours). A 42-hour discharge period allowed the introduced eDNA time to equilibrate and potentially bind to substrate and settle in the system allowing for realistic retention. Water sampling began approximately two hours after the water was emptied from the barrels (11:00 on Aug. 24). Surface water samples were collected approximately 15 cm below the surface in sterilized (10% bleach) 500 mL Nalgene bottles and stored them in sterilized coolers filled with ice. Sampling started at the source site (0 m) and we took 3 to 4 samples (sites that were shallower had 3 samples) from the center of the river every 60 m and facing upstream but walking backwards in a downstream direction to minimize sampling of kicked up sediment. We continued sampling downstream from the source site to 1.2 km downstream. Sampling took approximately 5.5 hours, finishing at approximately 16:30 on August 24. Water surface temperature was taken at each sample point and the mean was 16.8 °C (± SEM 0.2 °C). Positive control samples were

taken from the Atlantic Salmon holding tank in the FREC and negative samples were taken 40 m and 60 m upstream from the source site prior to downstream sampling. In total, 73 water samples were taken downstream from the source, four positive controls taken from the holding tank in the rearing facility, and six negative controls were taken upstream of the source. Three additional water samples were taken two days (~48 hours, at 08:30 on Aug. 26) after the initial completion of sampling at the source site to determine if trace amounts of Atlantic Salmon eDNA could still be detected.

2015 Experiment: The study was replicated in 2015 using the same set up and source site as 2014 (Fig. 2.1). All samples were collected and stored as above. However, in 2015 river control samples were collected prior to initiating the flow of the Atlantic Salmon water at the source site (0 m; N = 3), and 60 m (N = 3) upstream for a total of 6 river controls. In addition, three positive tank controls were collected from the Atlantic Salmon holding tank in the FREC. Atlantic Salmon water flow began at 14:00 on June 6, 2015 and stopped at approximately 7:00 on June 8, 2015 (total flow time: ~41 hours). Sampling began at 9:00 on June 8, two hours after the Atlantic Salmon water supply was exhausted. We began sampling 7.5 km downstream from the source site and moved upstream to the source site, collecting three surface water samples every 500 m with the bottle extended 60 cm upstream. We extended the sampling distance downstream in 2015 to determine if residual eDNA could be detected initially at greater downstream distances due to migration (Fig. 2.1). Sampling ended at 18:30 on June 8, approximately 9.5 hours after we started downstream sampling. Surface water temperature was taken at each sample point and the mean was 19.7 ^{o}C (± SEM 0.3 ^{o}C). In total, 48 water samples were collected, 6 negative controls, and three positive tank controls. We also included three additional water samples taken at the source site two days (~48 hours, at 08:00 on June 10) after initial sampling to determine if residual Atlantic Salmon eDNA can still be detected.

eDNA Filtering & Extraction

Within 24 hours of sampling, all water samples were filtered using Whatman® glass microfiber filter papers (47 mm diameter; 1.2 µm pore size; Whatman, Maidstone, UK). First, 500 mL of ddH_2O was filtered to act as blank controls to test for lab contamination (lab control hereafter), followed by the filtration of a river sample on a new filter paper on the same apparatus. This allowed each sample to have its own lab control and if any lab control tested positive for Atlantic Salmon DNA, the corresponding sample would not be used in our analysis due to potential contamination. Samples that were heavy with sediment required 2 to 3 extra filters (each with a corresponding lab control) and were included in qRT-PCR analysis. All filters were placed in 15 mL Falcon tubes and stored at -20 ^{o}C until DNA extraction.

For DNA extraction, each filter was cut approximately in half using sterile forceps and razor blades (cleaned between each use using 95% ethanol); one half was stored for future use, while the second half was used for eDNA extraction. Each filter paper section was cut into smaller strips to help with digestion and placed into 2 mL tubes containing 400 µL of 1.0 mm glass beads packed dry (Fisher Scientific LTD, BioSpec. Cat. No. 11079110), 400 µL of autoclaved ddH_2O, 400 µL of phenol-chloroform: isoamyl alcohol (25:24:1), and 400 µL of cetyl trimethylammonium bromide (CTAB) digestion buffer. The samples were homogenized for 3 minutes at 3000 strokes·minute^{-1} (speed setting 3 for the Mini-Beadbeater-24; Fisher Scientific LTD, BioSpec.) for complete digestion and mixing of the filter and contents. After centrifuging at 13 000 rpm for 20 minutes, the supernatant was removed for a second phase separation. We vortexed the supernatant with an equal volume of chloroform-isoamyl alcohol to remove any residual phenol in the solution, and centrifuged again for 20 minutes at 13 000 rpm. The supernatant was combined with equal volumes of isopropanol and 0.6X volume of 3M sodium acetate (pH 5.2), then left to precipitate DNA overnight at -20 ^{o}C. DNA was pelletized by

centrifugation at 13 000 rpm for 20 minutes, then supernatant discarded and pellet washed once with 70% ice cold ethanol. The DNA was pelletized again by a final centrifugation at 13 000 rpm for 20 minutes, ethanol discarded and sample pellets left to air dry for two hours to completely remove any residual ethanol. Once dry, DNA was re-suspended in 30 µL of 10 mM TE buffer and 0.5 µL of RNase A to eliminate any potentially interfering RNA. Samples were incubated at 37^{o}C for 1 hour to dissolve the DNA pellet. All DNA samples were stored at -20 ^{o}C until analysis.

Genetic Markers

We used primers that target the mitochondrial genome because of the stability of the circular mtDNA molecule and the high copy number present in each cell. We used Atlantic Salmon species-specific D-loop primers (Salmo_Mito-951) developed by Karlsson et al. (2012) to differentiate between Atlantic Salmon and Brown Trout (*Salmo trutta*). This primer set amplifies a DNA fragment size of approximately 230 bp (Karlsson et al. 2012). We used Primer Basic Local Alignment Search Tool (Primer-BLAST) against the Genbank database to check for primer specificity that returned hits only for *Salmo salar* or *Salmo trutta* (both species not found in Little River). Primer efficiency was tested using Atlantic Salmon DNA extracted from fin clips (i.e. positive PCR control) in a 10-fold dilution series (standard curve) and run in triplicate using qRT-PCR (see below for details of qRT-PCR).

qRT-PCR Analyses

All samples (except the qRT-PCR positive and negative controls) underwent a 10-fold dilution to decrease qRT-PCR inhibitors prior to analysis. qRT-PCR was run for each eDNA sample in triplicate in 20 µL reactions using 10 µL of SYBR® Select Master Mix (Life Technologies Inc.,

Burlington, ON, CA), 0.2 µM of each forward and reverse primer, 0.2 mg·mL^{-1} BSA, 0.1 units *Taq* polymerase, and 2.0 µL of template eDNA. No-template-controls and positive PCR controls were also included in triplicate for each qRT-PCR 96-well plate. Thermal cycler conditions were set to an initial 95 ^{o}C denaturation for 10 minutes, followed by 55 cycles of 94 ^{o}C denaturation for 30 seconds, and annealing at 60 ^{o}C for 60 seconds. All qRT-PCRs data were analyzed on the QuantStudio^{TM} 12K Flex Real-Time PCR System (Life Technologies Inc. Burlington, ON, CA).

Samples (biological and technical replicates) that did not produce a fluorescence signal were assigned a Ct value of 55 (maximum number of cycles). A subset of the qRT-PCR products was run on an agarose gel to confirm that a single band of the proper target size (~230 bp) was present. PCR products for six eDNA samples (three samples from 2014 and three samples from 2015) that produced clear bands were sequenced at the McGill University Gènome Québec Innovation Centre (Montréal, QC, Canada). A Basic Local Alignment Search Tool (BLAST) was used to align the resulting sequences against GenBank nucleotide database sequences for Atlantic Salmon and Brown Trout to verify amplification of the target species.

Data Analyses

To estimate primer efficiency, Atlantic Salmon DNA was analyzed via qRT-PCR with concentrations ranging from 32.0 ng·µL^{-1} to 3.20 x 10^{-9} ng·µL^{-1} and fitted to a logarithmic regression model. The DNA template concentrations were measured using Quant-iT^{TM} PicoGreen dsDNA assay (ThermoFisher Scientific, MA, USA) and verified using NanoVue spectrophotometry and gel analysis using a 2-fold dilution series of lambda DNA (ThermoFisher Scientific, MA, USA). The resulting standard curve was used to estimate the LOQ which corresponds to the lowest template DNA concentration that can be quantified (Armbruster & Pry 2008; Bustin et al. 2009). This value is calculated using the lower 95% confidence interval of the mean Ct ± SEM for the

no-template-control replicate and was set as our LOQ threshold (McKee et al. 2015); mean Ct values below this value are identified as positive for Atlantic Salmon DNA, and mean Ct values above this value are identified as negative. The mean Ct were calculated for each site by averaging the Ct values for all replicates per sample per site. We corrected the mean Ct values for all samples that were diluted (10-fold) for amplification using the slope obtained from the following qRT-PCR primer efficiency standard curve equation (Yun et al. 2006):

$$\text{Efficiency} = [10^{(-1/\text{slope})} - 1] \times 100\%$$

Finally, the mean Ct values per site were plotted against distance from the source site and fitted to a curvilinear regression model to test for a relationship between the variables. We expect residual eDNA signals to be stronger in sites closest to the source site (below LOQ), then remain fairly consistent with weaker signals at sites farther downstream.

Results & Discussion

Flowing aquatic ecosystems such as rivers can be problematic for the detection of a target species using eDNA analysis (Roussel et al. 2015). The flow of water not only physically moves target species' eDNA downstream away from their physical location, but the flow can also contribute to increased DNA degradation and dilution (Thomsen et al. 2012). This is especially problematic if eDNA concentrations in the system are already low, such as in studies focusing on invasive species at early stages in the invasion process, or rare species. Additionally, concentrations of detectable eDNA also decrease when target species leave the system (e.g. capture, predation, dispersal, or migration). Under such situations, eDNA studies rely on the detection of DNA molecules that have been retained in the system (i.e. residual eDNA). Residual eDNA are presumably eDNA molecules less subjected to environmental factors and thus decay at a slower rate. However, even longer lasting residual eDNA molecules will be lost over time

due to natural physical, chemical, and biological processes (Levy-Booth et al. 2007; Dejean et al. 2011) and, hence, its detectability will decline with time. Thus, the goal of this study was to study residual eDNA in a river after a known period of time since target species source removal.

In 2014, we sampled starting at the source site two hours after Atlantic Salmon water flow had ceased, and collected downstream with detections up to 960 m (Fig. 2.1). In 2015, we started sampling downstream from the source site and moved upstream up to the source site and had strong detections only at the source site (Fig. 2.1).

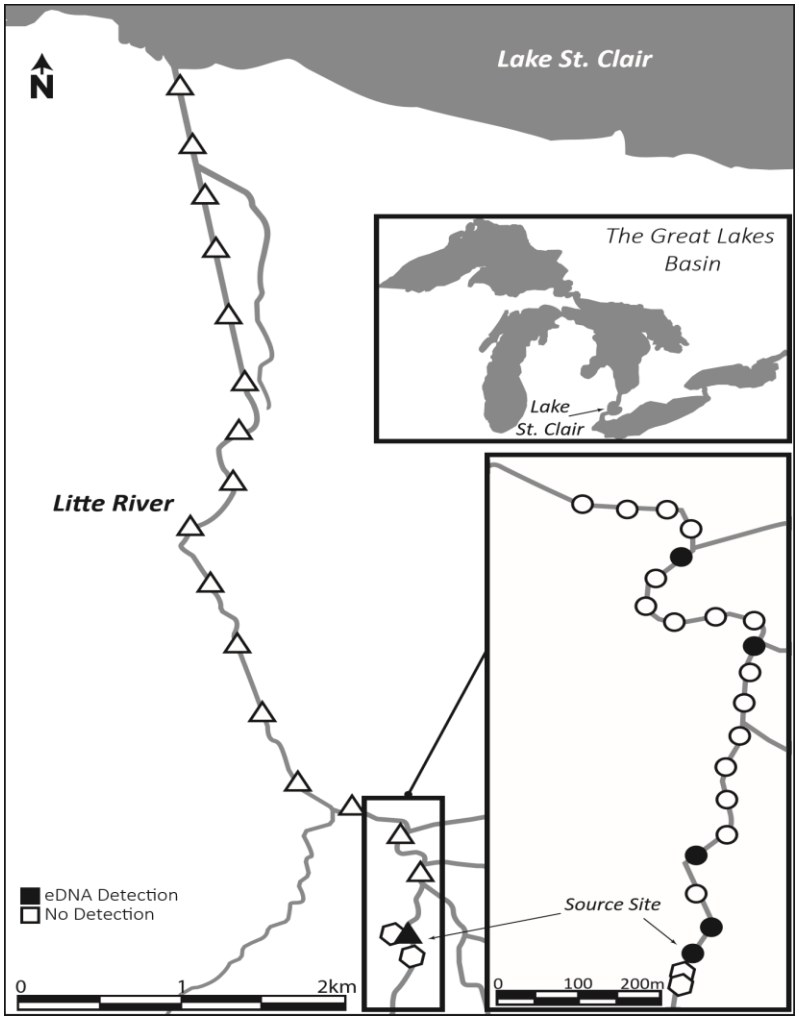

Figure 2.1. Little River eDNA sampling sites for 2014 (circles; N = 21) and 2015 (triangles; N = 16). Sampling in 2014 began at the source site (0 km) and moved downstream up to 1.2 km. Hexagons represent river controls. Sampling in 2015 began 7.5 km downstream and moved upstream towards the source site. eDNA detections represent sites with mean Ct values below our calculated LOQ threshold of 44.0 cycles.

Since our eDNA samples potentially contained inhibitors due to co-extraction of humic acids, fulvic acids, or organic matter (Hoshino & Inagaki 2012; Doi et al. 2015; McKee et al. 2015), all eDNA samples, including tank positives and river controls, underwent a 10-fold dilution prior to qRT-PCR amplification to minimize inhibitory effects and maintain consistency (McKee et al. 2015). Those mean Ct values were corrected using our qRT-PCR sensitivity plot producing a slope of -3.3 (R^2 = 0.97) with an overall efficiency of 101.0% (Fig. 2.2).

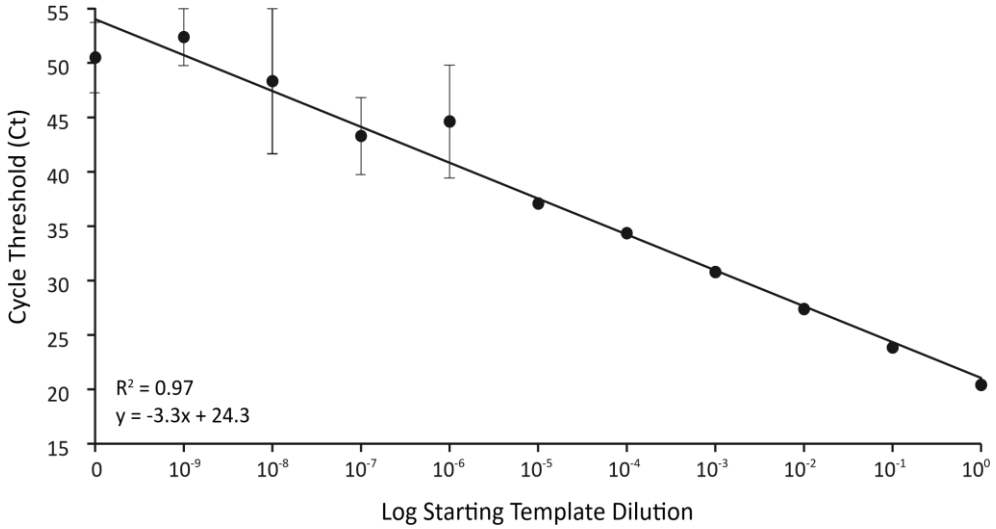

Figure 2.2. 10-fold PCR template DNA dilution series using the species-specific primer set Salmo_Mito-951 (Karlsson et al. 2012). Mean Ct (± SEM) values shown for each dilution triplicate with initial starting template concentration of 32.0 ng·µL^{-1}. LOQ at 44.0 cycles.

The LOQ threshold was 44.0 cycles, calculated using the lower 95% CI of our no-template-control with a mean Ct of 50.5 (± SEM 3.24) cycles. After Ct correction, our positive control samples taken from the Atlantic Salmon holding tank produced a mean Ct value of 31.5 (± SEM 0.6) cycles for 2014 and 34.1 (± SEM 0.7) cycles for 2015. In 2014, five sites produced mean Ct values below our LOQ threshold (0 m, 60 m, 180 m, 600 m and 960 m) with an overall positive curvilinear (cubic fit) relationship between distance from the source and mean Ct values (Fig. 2.3a; R^2 = 0.67, *df* = 17, *P* = 0. 00024). One replicate 2014 river control taken 40 m upstream from the source site was contaminated with Atlantic Salmon DNA (mean Ct of technical

replicates after correction was 39.7 (± SEM 6.0) cycles), however the rest of 2014 river controls were above our LOQ for Atlantic Salmon (Fig. 2.3a). The most likely explanation for the one contaminated upstream control sample is either backflow from the source site or field-based contamination since all lab controls were negative. Hence, in 2015 to avoid the possibility for field contamination, all river control samples were taken two days prior to field setup of the Atlantic Salmon water, and all 2015 river and lab controls were negative. In 2015, only one site (0 m) had a mean Ct value below our LOQ threshold, but there was an overall significant, curvilinear fit (Fig. 2.3b; $R^2 = 0.49$, $df = 12$, $P = 0.037$).

There were no eDNA detections below the LOQ threshold at the source site after 48 hours after species removal in either year (mean Ct of 49.0 (± SEM 2.5) cycles in 2014, and 50.3 (± SEM 1.4) cycles in 2015). By sampling surface water, we greatly reduced the potential of sampling sediment-bound eDNA which can result in false positives, since sediment-bound eDNA can persist much longer than water column eDNA (Turner et al. 2015). The mean Ct values for our 2014 samples had high associated standard errors (Fig. 2.3a) due to variability in quantification using qRT-PCR for highly dilute template DNA. If amplification failed and no quantifiable Ct value was produced within 55 cycles of our protocol, a Ct value of 55 was assigned to biological and technical replicates. Inclusion of those assigned Ct values increased the overall mean Ct value, which also inflated the error estimate. However, in 2015, the standard errors were lower and, on average, consistent between sites as the majority of samples had low template DNA concentrations. This was likely due to sampling beginning 7.5 km downstream from the source site and took 9.5 hours to reach the source site (11.5 hours after eDNA source removal), whereas in 2014 we began sampling at the source site and moved downstream. Beginning further downstream allowed more time to pass for residual eDNA to undergo dilution and degradation. Majority of biological and technical replicates within 2015

sites were assigned a Ct value of 55, reducing the error estimates, but producing mean Ct values above the LOQ. In both years, residual eDNA signals were greatest in sites towards the source site (below LOQ), then began to plateau towards more downstream distances (Fig. 2.3). This relationship implies that as downstream distance from the eDNA source increases, residual eDNA becomes more dispersed through the system resulting in lower concentrations of target residual eDNA being sampled. This could also represent the form of residual eDNA in aquatic systems, where eDNA can be present in clumps when released close to the source (Wilcox et al. 2015), and becomes more dispersed and randomized in the system as it migrates downstream.

It is possible to use residual eDNA signal strength based on qRT-PCR Ct values to infer the recent presence and approximate location of the target species. For example, in our study the source site is unambiguously identified by a greater drop in mean Ct value, even in 2015 where eDNA signals beyond the source site were above the LOQ (Fig. 2.3b). In 2014, more sites produced mean Ct values below the LOQ likely due to earlier sampling closer to the source site, producing samples with higher concentrations of target eDNA. Overall, the maximum distance that produced a positive residual eDNA signal in both years was 960 m downstream from the source site (Fig. 2.3a). The localization of the eDNA source was possible for the target species even after it had been removed. In our case, the eDNA source was removed, but in natural applications the target species may have moved out of the area, been captured and removed, or possibly consumed. Quantitatively mapping residual eDNA signals may be applicable for low-abundance species such as at-risk species or newly introduced species, as they are likely to be more localized to one area (e.g. critical habitat), mimicking a source site with eDNA signals declining downstream. Abundant species are more spatially dispersed and will continually replenish eDNA in the system, resulting in stronger and more consistent signals spread throughout the habitat and making it difficult to pinpoint precise species' locations.

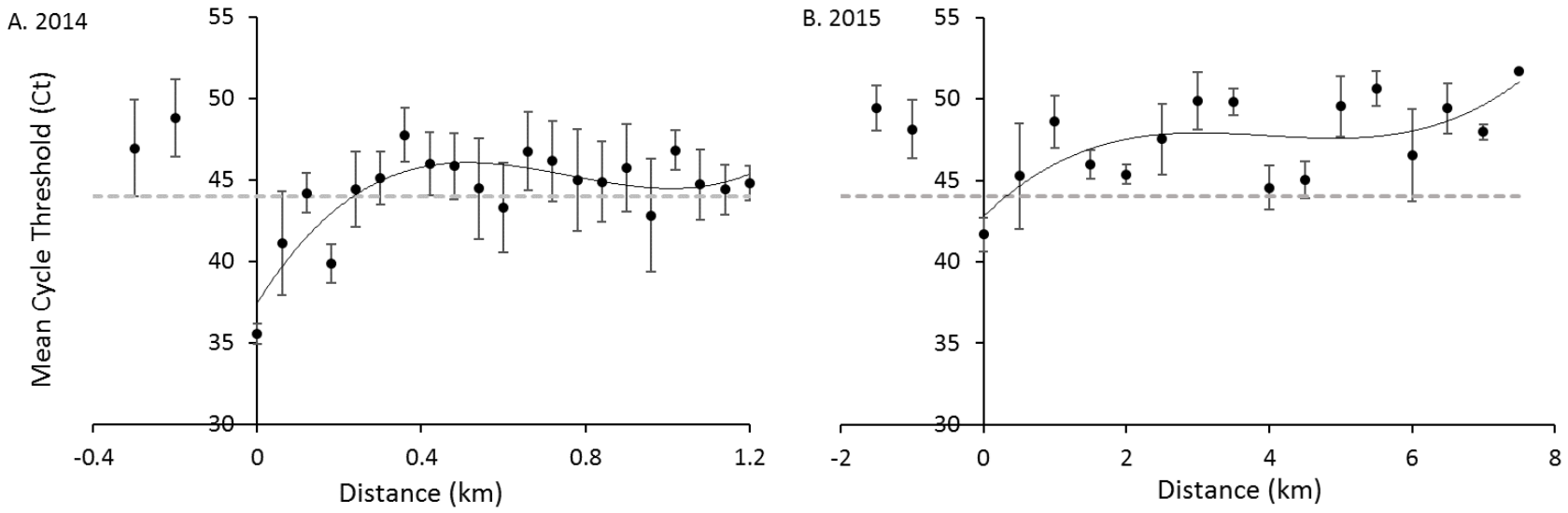

Figure 2.3. Cubic plot of mean Ct (± SEM) values against distance from eDNA source for (A) 2014, and (B) 2015 with each point showing sample sites in the Little River. Upstream river controls are shown as negative distances. Grey dashed line represents the LOQ threshold of 44.0 cycles. The solid lines are the fitted cubic regression lines for 2014 (A; $R^2 = 0.67$, *df* = 18, *P* = 0.00024) and 2015 (B; $R^2 = 0.49$, *df* = 12, *P* = 0.037).

Finally, we sequenced three PCR amplicons from the river samples for each year and sequences were confirmed to be Atlantic Salmon (Fig. 2.4).

Figure 2.4. Sequence alignment of 2014 and 2015 eDNA Little River mitochondrial D-loop PCR amplicons with known Atlantic Salmon (*Salmo salar*; GenBank accession: JQ390055.1) and Brown Trout (*S. trutta*; GenBank accession: M97984.1) sequences. Highlighted regions indicate base difference, dashes represent sequence gaps, and nucleotide N represents all bases or an unknown base call.

Overall, residual eDNA was detectable at the source site up to 11.5 hours after eDNA source removal in a flowing system. Based on laboratory mesocosms, Thomsen et al. (2012) reported detecting Common Spadefoot Toad (*Pelobates fuscus*) and Great Crested Newt (*Triturus cristatus*) eDNA up to 14 days after species removal, while Strickler et al. (2015) detected Bullfrog (*Lithobates catesbeianus*) eDNA up to 54 days after species removal; both studies used laboratory mesocosms. New Zealand Mudsnail (*P. antipodarum*) eDNA was detectable 21 days after species removal in laboratory aquaria (Goldberg et al. 2013), and Idaho Giant Salamander (*Dicamptodon aterrimus*) eDNA was detected up to 8 days with full sun exposure, and 11 days in shaded treatments (Pilliod et al. 2014). While our window of detection of residual eDNA was on the order of a few hours, our experiment is novel in that it examines residual eDNA detection after eDNA source removal within a natural, flowing ecosystem. Flowing ecosystems are often hotspots for species of conservation concern, as rivers hold greater diversity for fish species compared to lakes or ponds (Helfrich & Neves 2009), and act as invasion corridors for potential invaders (Yamanaka & Minamoto 2016). For example, the Round

Goby (*Neogobius melanostomus*) has spread throughout the Great Lakes and continues to spread via associated tributaries (Kornis et al. 2012). Asian carps (*Hypophthalmichthys* spp.) have a potential invasion corridor into the Great Lakes basin as a consequence of canals linking the Mississippi River to Lake Michigan (Jerde et al. 2011). Thus, the dynamics of eDNA in flowing ecosystems is critically important for identifying target species habitat, and for estimating how long eDNA can persist if the source is removed.

The ability to detect spatially localized rare aquatic species such as at-risk species or early aquatic invaders, is challenging when using conventional methods that have low detection probabilities (Taberlet et al. 2012; Thomsen & Willerslev 2014). The non-invasive eDNA approach offers an alternative sampling approach, capable of detecting very low quantities of residual eDNA. Once a target species is removed from the system, eDNA concentration will deplete rapidly as it is no longer replenished by the eDNA source (Barnes et al. 2014), thereby, reducing false positives. In flowing systems such as rivers, eDNA detection is even weaker due to downstream migration of eDNA molecules resulting in dilution and physical degradation (Fukumoto et al. 2015). We showed that residual eDNA can be used to provide a remarkably precise estimate of the target eDNA source location in an uncontrolled, flowing system, after the eDNA source had been removed from the system. The use of qRT-PCR is also more sensitive than traditional end-point PCR, decreasing false negative error rate (Thomsen et al. 2012; Wilcox et al. 2013; McKee et al. 2015). eDNA and qRT-PCR can be used as an initial survey tool in lotic systems to determine where the target species is located (and if it is still in the system), followed by management or further sampling efforts focused in appropriate sites, effectively saving time, costs, and conservation effort.

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CHAPTER 3

ENVIRONMENTAL DNA DETECTION OF INVASIVE AND RARE FISH SPECIES WITH THEIR ASSOCIATED FRESHWATER FISH COMMUNITY IN TWO GREAT LAKES TRIBUTARIES[2]

Introduction

Environmental DNA (eDNA) methods have been shown to have higher sensitivity in detecting rare species when compared to conventional methods, by extracting DNA from environmental samples (Ficetola et al. 2008; Taberlet et al. 2012; Jerde et al. 2011; Jerde et al. 2013; Dejean et al. 2012; Nathan et al. 2014). Non-invasive detection methods based on eDNA are particularly valuable for species at risk or early detections of non-native and invasive species (Bohmann et al. 2014; Barnes & Turner 2016). Species at risk are judged as endangered, threatened, or extirpated according to the *Species at Risk Act* (SARA; SARA 2016) and are thus present at a low abundance (i.e. rare). eDNA analysis has been successfully used in rare aquatic species detection of fishes (Laramie et al. 2014; Sigsgaard et al. 2015), amphibians (Fukumoto et al. 2015; Spear et al. 2015; Pierson et al. 2016), reptiles (Davy et al. 2015), and invertebrates (Goldberg et al. 2013; Mächler et al. 2014). With successful eDNA detections of species with low abundance, eDNA applications have expanded to encompass invasive species early in their invasion (i.e. still present in low abundance). Recent work on early invasive species detection using eDNA methods include the detection of invasive Common Carp (*Cyprinus carpio*) haplotypes in Japan (Uchii et al. 2016), Bluegill (*Lepomis macrochirus*) in Japanese ponds (Takahara et al. 2013; Doi et al. 2015), Burmese Python (*Python bivittatus*) in Florida, USA (Piaggio et al. 2013), and monitoring of multiple invasive fish species in the Great Lakes bait

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trade (Nathan et al. 2014). Hence, eDNA is useful for the detection of rare species and the early detection of invaders, allowing for earlier implementation of eradication or control programs (Anderson 2005; Hulme 2006).

While eDNA has been used extensively for determining the distribution of target species, simple presence data does not provide an ecological context for conservation, management, and invasion risk assessment efforts. Sampled water will contain DNA from all inhabitants in the system that can be utilized to characterize whole communities (Yamanaka & Minamoto 2016). Community-level eDNA analyses are based on metabarcoding, which uses eDNA to detect multiple species simultaneously by massively parallel (e.g. next-generation) DNA sequencing to detect and identify all species in the community (Pompanon et al. 2011; Taberlet et al. 2012; Thomsen et al. 2012; Goldberg et al. 2015; Port et al. 2016). Moreover, gathering presence data for the whole community indirectly monitors for unknown species (e.g. recently introduced species) as a passive surveillance of the system (Simmons et al. 2016). As an example, Port et al. (2016) used NGS and multiple species targeted primers to amplify eDNA extracted from marine kelp forests, and detected several species which included cryptic species that were often missed by conventional methods. Hence, the use of multiple targeted and generic primers, coupled with next-generation sequencing (NGS) of eDNA samples, can be used simultaneously detect rare, cryptic, elusive, newly introduced species and common natives, generating useful data for whole community analysis relevant to species of interest.

As a novel method, eDNA analyses of target species distribution and community composition are powerful, but results must be interpreted with caution. eDNA production and degradation rates are affected by biotic factors such as fish biomass (Klymus et al. 2015), life stage (Maruyama et al. 2014), spawning season (Fukumoto et al. 2015; Spear et al. 2015), and abiotic factors such as turbidity (Diffey et al. 2002), and flow rate affecting eDNA migration,

settlement, and physical and chemical degradation (Takahara et al. 2012; Deiner & Altermatt 2014; Turner et al. 2015). Sampling protocols, DNA extraction methods, and different eDNA analyses have also influenced species detection results (Matheson et al. 2010; McKee et al. 2015; Takahara et al. 2015). The potential for false positive and/or negative outcomes from eDNA detection analyses must be evaluated carefully; however, improved protocol design and methodology has demonstrated eDNA analysis to be reliable (Jerde et al. 2011; Dejean et al. 2012; Rees et al. 2014; McKee et al. 2015).

Effective management and conservation of aquatic ecosystems require data on the distribution of rare and invasive species. However, the inclusion of community composition data provides an ecological perspective on species at risk spatial patterns in relation to the presence of other native or invasive species. In this study, we characterize the spatial distribution of three species at risk, one invasive species, and native species in two large Great Lakes tributaries using eDNA metabarcoding methodology. We combined slightly more targeted species PCR primers (species primers hereafter) with a community PCR primer designed to target all co-occurring fishes in the study systems. We use NGS to maximize data gathered on the spatial relationship between SAR, invasive species, and the native fish community composition. Our combined eDNA/NGS approach is compared to capture-based methods previously conducted in the same study rivers to validate eDNA analysis as an effective conservation tool. The results in this study will shine light on the efficacy of eDNA analysis to simultaneously provide presence/absence and spatial distribution of rare, invasive, and common native species in two large Great Lakes tributaries.

Materials & Methods

Study Area

The Sydenham River is a tributary of Lake St. Clair (refer to Fig. 3.2), which historically had a high level of aquatic biodiversity; approximately 80 fish species (Staton et al. 2003; Marson & Mandrak 2009). Currently, 51 fish species have been recently reported in lower East Sydenham River (SCRCA 2013b). The Sydenham River has two main; the North Sydenham River which is approximately 70 km long with a discharge of 16.9 $m^3 \cdot s^{-1}$, and the East Sydenham River which is approximately 100 km long with a discharge of 34.4 $m^3 \cdot s^{-1}$. The total catchment drainage area of both branches is 2725 km^2 (Metcalfe-Smith et al. 2003; Staton et al. 2003).

The Grand River drains into Lake Erie (refer to Fig. 3.2) and is the largest watershed in southern Ontario, approximately 300 km long with catchment drainage area of 6800 km^2 (Li et al. 2016). It has an annual mean flow rate of approximately 64 $m^3 \cdot s^{-1}$ (Singer & So 1980). The watershed is home to more than half of all freshwater fishes in Ontario (158 species; Mandrak & Crossman 1992), with a report of 83 species found to be present in Grand River in 1999 (Wright & Imhof 2001).

Study Species

The four target species in this study include three species at risk; Northern Madtom (*Noturus stigmosus*), Eastern Sand Darter (*Ammocrypta pellucida*), and Silver Shiner (*Notropis photogenis*), and one invasive species, the Round Goby (*Neogobius melanostomus*). The target species at risk have overlapping historical or current distributions in at least one of the two study rivers. The invasive Round Goby is known to be present at high densities at many locations in both study rivers, with known detrimental impacts on many native and at-risk species (Poos et al. 2010; DFO 2011; DFO 2012).

The Northern Madtom (Siluriformes: Ictaluridae) is a small (120 mm) benthic, cavity-nesting catfish listed as Endangered under SARA (SARA Schedule 1 2016). It prefers large creeks and big rivers with slightly turbid waters and bottoms comprised of sand, gravel, and silt for nesting (DFO 2012). The Canadian distribution is restricted to Lake St. Clair, St. Clair River, Detroit River, Thames River, and possibly in the Sydenham River; however, they have not been collected in Sydenham River since 1975 (COSEWIC 2012; DFO 2012). The Northern Madtom is not known to be present in the Grand River either currently or historically. The main threats to Northern Madtom include habitat degradation such as nutrient loading and habitat fragmentation, and competition with invasive species such as the Round Goby for food and habitat space (Edwards et al. 2012).

The Eastern Sand Darter (Perciformes: Percidae) is a small (71 mm), benthic fish and the only member of the genus *Ammocrypta* found in Canada (COSEWIC 2009). It is listed as Threatened by SARA (SARA Schedule 1 2016). Its distribution in Canada is limited to Lake Erie, Lake St. Clair, Sydenham River, Thames River, Grand River, Big Creek, and Big Otter Creek (likely extirpated) (DFO 2011). Eastern Sand Darter populations in Grand River were deemed “good” by DFO, and more than 735 individuals have been captured since 1987 when it was first reported (DFO 2011). Populations in Sydenham River were deemed “poor” with only 43 individuals recorded in the last two decades (DFO 2011) and only 12 caught in a more recent study (Ginson et al. 2015). Eastern Sand Darter spawning occurs in late spring, and inhabits lakes and shallow streams or rivers, with sandy bottoms for burrowing (COSEWIC 2009). Main threats to Eastern Sand Darter populations are habitat degradation caused by eutrophication and pollution from agricultural practices and urban development, invasive species (including Round Goby), and disease (DFO 2011; Finch et al. 2013).

The Silver Shiner (Cypriniformes; Cyprinidae) is a small (143 mm) fish with a known Canadian distribution consisting of four populations in Grand River, Sixteen Mile Creek, Thames River, and Bronte Creek. It is assessed as Special Concern by SARA (SARA Schedule 3 2016). Current literature does not indicate any present or historic populations of Silver Shiner in the Sydenham River, although a single individual was reportedly caught in the east Sydenham River in 2003 (DFO 2013). There are records of one "fair" population in Grand River with only a few individuals collected (DFO 2013). Silver Shiner prefer fast flowing systems such as large streams or deep riffles with moderate gradients and sand, clay and gravel substrates (McKee & Parker 1982). Breeding season is from April to June and they feed at the surface on algae and small aquatic invertebrates such as worms (COSEWIC 2011). The main threat to Silver Shiner populations is habitat degradation via contamination from poor water management (DFO 2013).

The Round Goby (Perciformes: Gobiidae) originally arrived via ballast water from Eastern Europe, with initial reports in Lake St. Clair in 1990 (Jude et al. 1992). It is a small (45 mm), highly invasive, benthic species that has spread throughout the Great Lakes and is expanding rapidly upstream in tributaries (Poos et al. 2010; Bronnenhuber et al. 2011). The impact of Round Goby on Silver Shiner is not known (DFO 2013). Round Goby burrow in rocky substrate or open crevices to lay eggs during the spawning season from April to September directly competing with Northern Madtom and Eastern Sand Darter for benthic habitat space (Kornis 2011; Kornis et al. 2012b; Edwards et al. 2012). It feeds on mussels (e.g. Dreissenidae), insect larvae, and young-of-the-year of native species such as Northern Madtom (DFO 2012; Burkett & Jude 2015). Furthermore, Round Goby feed nocturnally which also directly competes with Northern Madtom for foraging space (COSEWIC 2002; DFO 2012).

Capture Records

We used fish-capture records (collected by Fisheries and Oceans (DFO) Canada) of sampling conducted in 2010 to 2012 during June and August for the Grand River, and from 2010 to 2015 during June, August, and September for the Sydenham River. A positive catch record requires at least one live individual caught at the sample site. The majority of sites in the Grand River were sampled using a Missouri trawl via boat (small 1/8" outer covering (2.5 m) or 8' foot rope with 24'x12' doors), although one site (G1) was sampled using a bag seine (dimensions: 1/8" bag mesh with 1/8" wing mesh, length 10 m to 30 ft). Fishes were caught using a variety of gear in the Sydenham River, including Missouri trawling, backpack electrofishing, bag seining, mini-fyke netting, gill netting, and trap netting. The DFO catch data was used to generate river-specific fish species lists for use in analyzing NGS data (67 species in total). In Sydenham River, the mean catch per unit effort (CPUE) was 187.7 (± SEM 21.0) fish per haul using Missouri Trawl, 51.7 (± SEM 13.7) fish per haul using bag seines, 12.5 (± SEM 6.9) fish per minute via backpacking, and 0.16 (± SEM 0.12) fish per minute using a combination of gill nets, trap nets, mini fyke nets, and trammel nets. In Grand River, the mean CPUE was 77.4 (± SEM 8.1) fish per haul/trawl via Missouri Trawl, and 116.7 fish per haul using bag seines (refer to Table 3.3 for catch numbers per species).

Water Sampling

Sampling was conducted in September 2013 for Grand River and in October 2013 for Sydenham by DFO using a boat. We used a modified Jerde et al. (2011) sampling protocol. All field equipment was sterilized by soaking in 10% bleach for 10 minutes and rinsed using double-distilled water (ddH_2O). Water samples from 44 sites in the Sydenham River and 43 sites in the Grand River were collected in 500 mL Nalgene bottles and stored in the field in coolers

containing ice. Surface samples were collected just under the surface, while samples collected at greater depths were characterized as bottom samples. Blank river site control samples (river controls hereafter) were included at a subset of sample sites (13 sites for Grand River and 10 sites for Sydenham River) by opening a 500 mL Nalgene bottle filled with ddH_2O to expose the contents to the air, sealed, and stored in the cooler alongside the river water samples. The majority of samples collected by DFO at each site were replicated (2-3 bottom samples and 10-15 surface samples) and we used a subset of 2-5 samples per site for eDNA analysis. In total, we used 184 Sydenham River samples (111 surface, 70 bottom, and 3 river controls) and 170 Grand River samples (108 surface, 58 bottom, and 4 river controls).

eDNA Extraction

Within 24 hours of sampling, all water samples were filtered using Whatman® glass microfiber filter papers (47 mm diameter; 1.2 µm pore size; Whatman, Maidstone, UK). Prior to filtering each river sample, 500 mL of ddH_2O was filtered on a separate filter to act as lab blank controls (lab control hereafter), followed by the filtration of the river sample on a new filter using the same filtration apparatus. This allowed each sample to have its own lab control – we also included lab controls for the river control samples. If any lab control tested positive for target DNA during PCR amplification, the corresponding sample would be excluded due to potential contamination (i.e. false positives). If a river sample had a high sediment load, we used up to four separate filters, each with its own lab control. Each filter was placed in a 15 mL Falcon tube and stored at -20 ^{o}C until DNA extraction.

For DNA extraction, filters were cut into halves using sterile forceps and razor blades cleaned between each use using 95% ethanol (note: lab and river controls were done on a separate day to avoid accidental cross-contamination). One half of the filter was stored and the

second half was used immediately for DNA extraction. The half filters were cut into strips to help with digestion and placed into 2 mL screwcap tubes containing 400 µL of 1.0 mm glass beads packed dry (BioSpec Cat. No. 11079110), 400 µL of ddH_2O, 400 µL of phenol-chloroform: isoamyl alcohol (25:24:1), and 400 µL of cetyl trimethylammonium bromide (CTAB) digestion buffer. The tubes were homogenized for two minutes at 3000 strokes per minute using Mini-Beadbeater-24 (Fisher Scientific LTD, BioSpec.) to allow complete cellular breakdown and protein digestion. The samples were centrifuged at 13 000 rpm for 20 minutes and the supernatant was transferred to a new 1.5 mL Eppendorf tube, and then vortexed with equal volumes of chloroform-isoamyl for a second phase separation. The mixed solution was centrifuged again and the supernatant was transferred to a new 1.5 mL Eppendorf tube, then mixed with an equal volume of isopropanol and one tenth volume of 3M sodium acetate (pH 5.2). The mixtures were left overnight at -20 ^{o}C. DNA was pelletized by centrifugation and washed once with 70% ice-cold ethanol and re-suspended in 30 µL of 10 mM TE Buffer and 1.0 µL of 20 $mg \cdot \mu L^{-1}$ RNase A to eliminate RNA present in the sample. All extracted eDNA was stored at -20 ^{o}C until further analysis.

Primer Design

Our design was to multiplex targeted species PCR primers with a broad fish community PCR primer set developed to amplify additional native species that inhabit the two Great Lakes tributaries (based on DFO catch data). COI sequences were collected from NCBI Genbank and Barcode of Life Database (BOLD; Ratnasingham & Hebert 2007) and aligned using Geneious v. 6.1 (Biomatters, www.geneious.com). All primers were constructed with 5' tails: Uni-A (forward) and Uni-B (reverse) for NGS library preparation (Table 3.1).

Table 3.1.

List of species targeted and fish community primer set sequences developed in this study for the detection of rare, invasive, and common native species from environmental DNA. Associated average melting temperature (T_M), and fragment size (bp). Annealing temperature used for all primer sets was 52°C.

Species common name (*Scientific name*)	Primer Name	Sequence 5'-3'	Fragment Size (bp)	T_M (°C)	Ref
Round Goby (*Neogobius melanostomus*)	RG-COI-F	ACCTGCCTGCCGGGGGGAYGACCARATYTAT	381	78.0	This Study
	RG-COI-R	ACGCCACCGAGCGCGGGGGGYTTYATATTT		80.4	
Eastern Sand Darter (*Ammocrypta pellucida*)	ESD-COI-F	ACCTGCCTGCCGATCTAGTATTTGGTGCTTG	434	76.9	This Study
	ESD-COI-R	ACGCCACCGAGCATTAATGGCCCCTAGAATTG		80.4	
Silver Shiner (*Notropis photogenis*)	SS-COI-F	ACCTGCCTGCCGCGCTTTAAGCCTCCTTATTCG	396	82.4	This Study
	SS-COI-R	ACGCCACCGAGCTTAACTGCYCCTAGAATTG		76.2	
Northern Madtom (*Noturus stigmosus*)	NMT-COI-F	ACCTGCCTGCCGTTTCTTTATAGTAATACCAG	258	71.3	This Study
	NMT-COI-R	ACGCCACCGAGCAGGTGAAGGGAGAAGATGGTTAG		82.4	
	FishCom-COI-F	ACCTGCCTGCCGTATTTGGYGCYTGRGCCGGRATAGT	247	85.0	This Study
	FishCom-COI-R	ACGCCACCGAGCCARAARCTYATRTTRTTYATTCG		62.3	

Note: Uni-A tail attached to 5' end of forward primers. Uni-B tail attached to 5' end of reverse primers.

Species primers were designed to amplify the four target species and closely related species. Target and non-target species sequences will be unambiguously identified using NGS. Degenerate base sites were avoided within 5 bp of the 3' end to minimize mispriming (Kwok et al. 1990; Epp et al. 2012). Primer-BLAST (Ye et al. 2012) and Net Primer (Premier Biosoft, www.premierbiosoft.com/netprimer) were used to verify primer specificity, low self-complementarity, uniform annealing temperatures, no secondary structure, and appropriate fragment length. Each species primer pair was tested using target species DNA and primer efficiency was assessed using a 10-fold dilution series of known DNA template concentrations run in triplicate with SYBR Green qRT-PCR; concentrations ranged from 10 $ng \cdot \mu L^{-1}$ to 10^{-6} $ng \cdot \mu L^{-1}$. No-template-controls were included in triplicate in the same qRT-PCR plate. qRT-PCR conditions were set to an initial denaturation at 95 ^{o}C for 10 minutes, followed by 45 cycles of 95 ^{o}C for 15 seconds and 60 ^{o}C annealing temperature for 60 seconds. The limit of detection (LOD) at which the lowest DNA concentration that can be observed (Armbruster & Pry 2008; Kim et al. 2014), was analyzed using the logarithmic regression for the 10-fold dilution series per primer set. Next, species primers were tested for primer interference by combining all four primer sets in one multiplex reaction, which consisted of 0.5 µM of all forward and reverse primers, 25 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 $mg \cdot mL^{-1}$ BSA, 0.1 units of *Taq* polymerase, 2.0 µL of target species DNA template (separately for each target species) and ddH_2O for a total reaction volume of 25 µL. An extra PCR reaction was conducted using 0.5 µL of each target species DNA combined into a single PCR well to test if multiple DNA templates interfered with amplification, or produced non-target bands. PCR conditions were set to an initial denaturation for 2 minutes at 95 ^{o}C, followed by 40 cycles of 30 seconds at 94 ^{o}C, 30 seconds at 52^{o}C annealing temperature, 30 seconds extension at 72 ^{o}C, and a final extension for 10 minutes at 72 ^{o}C and held at 4 ^{o}C. PCR products were analyzed on 2% agarose gel and checked for one,

bright band of the correct amplicon size for each target species. The combined target species DNA reaction was analyzed for one thick, bright band that encompassed Round Goby, Eastern Sand Darter, and Silver Shiner, as well as one slightly smaller band for Northern Madtom.

Next, we developed a community primer set (PS1 hereafter) using COI sequences obtained from Genbank and BOLD for 67 fishes (including target species, refer to Table 3.3) captured in previous years in the two rivers by conventional methods. We determined the primer-template mismatch for all species from both rivers to estimate expected PCR amplification efficiency. Highly conserved regions surrounding polymorphic regions were selected for primer design, targeting a fragment size of ~250 bp. We avoided degenerate bases within 5 bp of the 3' end for all primers to reduce mispriming (Kwok et al. 1990; Epp et al. 2012). PS1 efficiency was tested against all four target species using a 10-fold series dilution and qRT-PCR. Dilution series for each target species used template DNA concentrations ranging from 10 $ng \cdot \mu L^{-1}$ to 10^{-6} $ng \cdot \mu L^{-1}$ in triplicate. No-template-controls were also included in triplicate in the same qRT-PCR plate. qRT-PCR reactions and LOD determination were conducted as above. For all primer sets, initial DNA template concentrates were 38.4 $ng \cdot \mu L^{-1}$ for Round Goby, 35.4 $ng \cdot \mu L^{-1}$ for Silver Shiner, 46.4 $ng \cdot \mu L^{-1}$ for Eastern Sand Darter, and 30.0 $ng \cdot \mu L^{-1}$ for Northern Madtom.

eDNA PCR

Multiplexed target species PCRs and PS1 PCRs were conducted separately using the same conditions. PCR reactions consisted of 2.5 µL of 10X *Taq* reaction buffer, 25 mM $MgCl_2$, 0.5 µM of each forward and reverse primer, 0.2 mM of each dNTP, 0.2 $mg \cdot mL^{-1}$ BSA, 0.1 units *Taq* polymerase, 1.0 µL of eDNA sample, and ddH_2O for a total reaction volume of 25 µL. PCR conditions were set to an initial denaturation at 95 ^oC for 2 minutes, then 40 cycles of 30 seconds at 94 ^oC, 30 seconds annealing temperature at 52 ^oC, 30 seconds at 72 ^oC for extension,

final extension at 72 °C for 10 minutes, and a final hold at 4 °C. Five PCR controls (4 target species benchmark DNA and a no-template-control) were also included for each prepared mastermix.

Next-Generation Sequencing Preparation

After eDNA PCR amplification, each eDNA sample had a total of two PCR products. We combined 15 µL of target species PCR product and 10 µL of PS1 PCR product per sample for a total of 25 µL combined PCR product. The combined PCR products were cleaned using Agencourt AMPure XP (Beckman Coulter, Mississauga, ON, Canada) to remove primer dimer and fragments less than 100 bp. Unique barcodes were attached to the PCR amplicons using a second, short-cycle PCR for NGS library preparation; library preparation thus required two rounds of PCR. Second round PCRs consisted of 2.5 µL of 10X *Taq* reaction buffer, 25 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM P1+Uni-B adaptor (5'-CCTCTCTATGGGCAGTCGGTGATacgccaccgagc-3'), 0.5 µM A+barcode+key+Uni-A adaptor (5'- CCATCTCATCCCTGCGTGTCTCCGACTCAGxxxxxxGATacctgcctgccg), 0.1 units *Taq* polymerase, 10 µL of cleaned PCR product, and ddH_2O for a total reaction volume of 25.5 µL. Short-cycle PCR conditions started with a 2-minute denaturation at 95 °C followed by 6 cycles of 95 °C denaturation for 30 seconds, 60 °C annealing temperature for 30 seconds, 72 °C extension for 30 seconds, and a final extension at 72 °C for 5 minutes. Second round PCR products with attached adaptors were purified again using Agencourt AMPure XP, 10 µL of each sample combined in a 1.5 mL tube (72 samples per tube), and precipitated at -20 °C overnight using equal volumes of isopropanol and one tenth volume of 3M sodium acetate (pH 5.2). The combined PCR products (i.e. library) was centrifuged, isopropanol discarded, and the pellet was washed once with 70% ice-cold ethanol. The tubes were centrifuged again, ethanol discarded, and the library was

eluted in 50 µL of ddH_2O. Next, 20 µL of the library was gel extracted and bioanalyzed to determine final DNA concentration using Agilent High Sensitivity DNA chip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). Finally, the library was diluted to a final concentration of 55 $pmoL \cdot L^{-1}$ and sequenced on a 318 chip (approximately 4 million reads) using the Ion Chef™ System (Life Technologies, USA).

Analyses

NGS data was processed using Quantitative Insights into Microbial Ecology (QIIME) software (Caporaso et al. 2010) to remove sequences that were smaller than 200 bp, de-replicate sequences, remove sequences with more than three primer-template mismatches, or do not meet the default minimum average quality score of 25 (corresponds to an average error rate of 1%, or 99% accuracy in the accepted reads). We used the fish species COI sequences previously used for PS1 primer design to create custom reference database for each river. The filtered NGS sequences were compared against the custom database using BLASTn with default parameters changed from an expected (E) value of 10 to 10^{-60} and percent identity from 0% to 96% to ensure high confidence in the returned species identification. We classified a species as "present" at a site only if it returns at least three eDNA sequence matches in a sample. Disregarding one hit (singletons) or two hits (doubletons) avoids diversity overestimation produced by PCR or sequence artefacts, but accepting eDNA matches of three allows rare sequences to be counted; an important consideration for rare species (Zhan et al. 2014). Due to the rarity of our species at risk, we explored all positive species at risk eDNA sequence reads for agreement between bottom and surface samples to determine which sample type produced more positive eDNA results. We expected our benthic at-risk species (Eastern Sand Darter, and

Northern Madtom) to contribute more eDNA in bottom samples than surface samples, and the opposite for Silver Shiner.

The eDNA presence data for the target species at risk was mapped to display their spatial distribution, and we compared the eDNA presence data with species at risk spatial distribution based on conventional methods. As some sites sampled for eDNA analysis were not sampled by DFO, comparison between the two methods only includes the overlapping sites (18 sites in Sydenham River and 29 sites in Grand River). The two sampling methods were statistically compared using the McNemar chi-square test, which tests for differences in detection between two sampling methods for the same species, sampled in the same study rivers (McNemar 1947). Next, whole community analysis of species co-occurrences was analyzed using R Studio v. 0.99.892 (RStudio Team 2015) with the package "cooccur" (Veech 2013; Griffith et al. 2016) to determine positive, negative, and random (i.e. no significance) spatial co-occurrence distributions between species using the default settings of the package, except the initial threshold was set to FALSE instead of TRUE to analyze all possible combinations without excluding species that are not expected to have more than 1 co-occurrence (this avoids excluding rare species co-occurrences).

Results

Primer Validation

Target species PCRs showed uniform amplification efficiency (Fig. 3.1a). The species primer sets had the following primer efficiencies: Round Goby (R^2 = 0.96), Eastern Sand Darter (R^2 = 0.99), Northern Madtom (R^2 = 0.95), and Silver Shiner (R^2 = 0.96). Target species PCRs also showed uniform amplification efficiency for PS1 (Fig. 3.1b). Based on the regression curve of

each primer set for each target species, the limit of detection flattened at template dilutions of 10^{-5} ng·µL^{-1}.

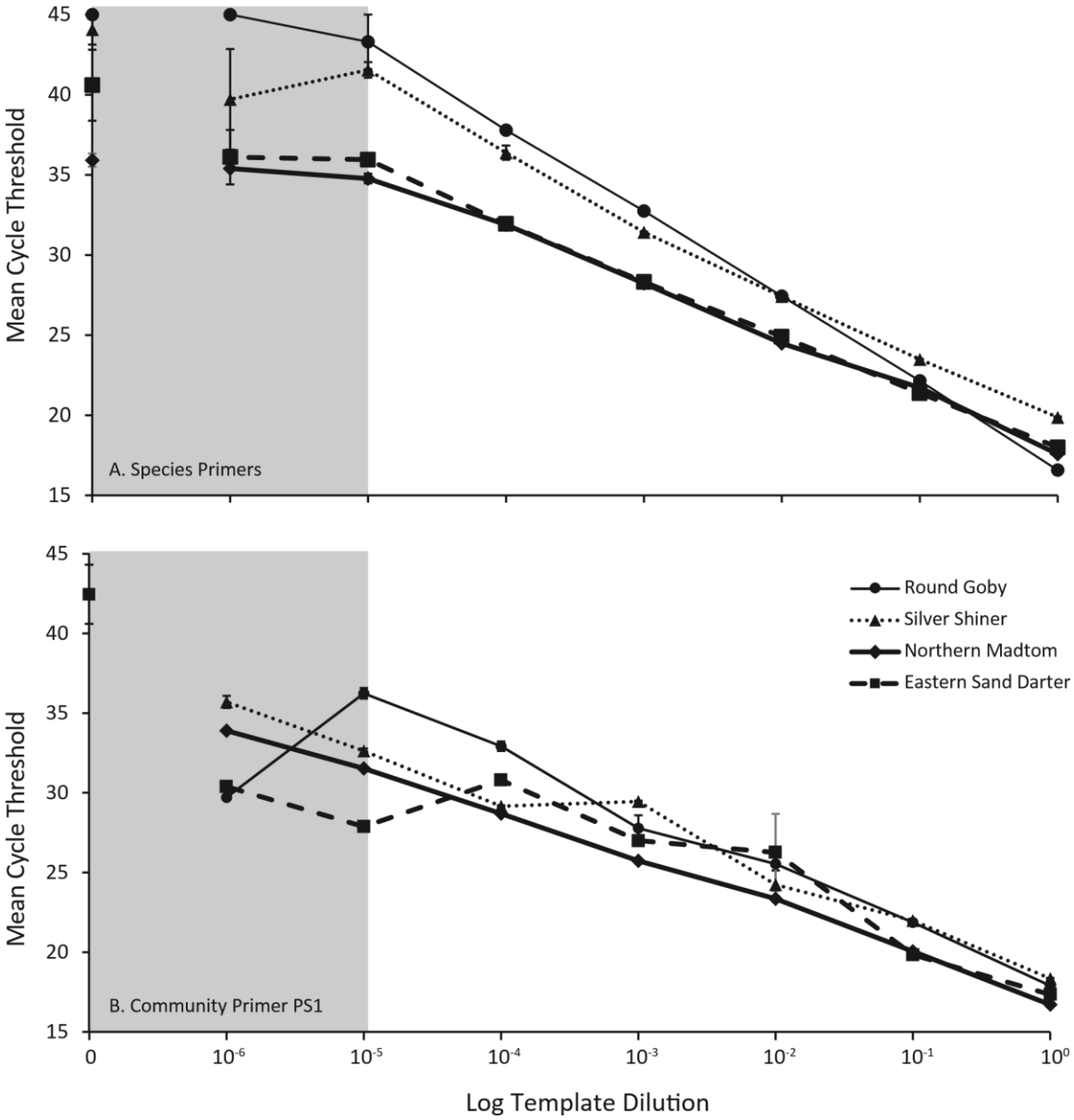

Figure 3.1. 10-fold PCR template dilution series using qRT-PCR for (A) species primers and (B) fish community primer PS1. Mean Ct (± SEM) is shown for each dilution (qRT-PCR done in triplicate), including the no-template-control shown as zero. Initial DNA template concentrations were 38.4 ng·µL^{-1} for Round Goby, 35.4 ng·µL^{-1} for Silver Shiner, 46.4 ng·µL^{-1} for Eastern Sand Darter, and 30.0 ng·µL^{-1} for for Northern Madtom. Shaded region represents the plateau of the dilution curve and hence the limit of detection.

Next-Generation Sequence Data

Our next-generation sequencing produced 6.5 million raw reads for the target species PCR cocktail and PS1 library. Primer PS1 detected more species in more samples from both rivers compared to multiplexed species primers (Table 3.2).

Table 3.2.
Summary of eDNA NGS data for multiplexed species primers and community primer PS1 for samples with ≥ 3 eDNA sequence matches to a species (numbers in front of square brackets). Numbers in square brackets indicate the total number of samples or sites used in this study. In total, 170 Grand River samples from 43 sites and 184 Sydenham River from 44 were analyzed.

Primer Set	River Controls	Bottom Samples	Surface Samples	Species Detected	Sites with positive eDNA detections
Grand River					
Multiplex Species Primes	0 [4]	1 [58]	3 [108]	2 [67]	4 [43]
Community Primer: PS1	0 [4]	53 [58]	105 [108]	43 [67]	43 [43]
Sydenham River					
Multiplex Species Primes	0 [3]	0 [70]	0 [111]	0 [67]	0 [44]
Community Primer: PS1	1 [3]	69 [70]	104 [111]	42 [67]	43 [43] [a]

Note: [a] total sites reduced to 43 from 44 due to exclusion of site S16.

Multiplexed species primer sets: After quality control NGS resulted in 3311 reads using the Round Goby primer set with an average of 11.3 sequences per sample (range = 1 to 67 reads/sample), for 292 samples (130 Grand River and 134 Sydenham River samples). After BLASTing, there were eight matches to Round Goby in 2 samples from Grand River and 5 samples from Sydenham River, and were excluded due to less than 3 eDNA matches per sample. Additionally, there was one match for Bluntnose Minnow (*Pimephales notatus*) in one Sydenham River sample. The Silver Shiner primer returned 10 876 reads in 318 samples (147 Grand River and 142 Sydenham River) with an average of 34.2 sequences per sample (range = 1 to 276 reads/sample). The Silver Shiner species primer returned several related cypriniform species including Bluntnose Minnow (*Pimephales notatus*), Common Carp (*Cyprinus carpio*), Common Shiner (*Luxilus cornutus*), Emerald Shiner (*Notropis atherinoides*), Ghost Shiner (*Notropis buchanani*), Mimic Shiner (*Notropis volucellus*), Rosyface Shiner (*Notropis rubellus*),

Northern Hogsucker (*Hypentelium nigricans*), and Round Goby. Hence, despite many sequence reads, there were only two sequence matches for Silver Shiner. The Eastern Sand Darter species primer returned 24 967 reads in 339 samples (150 Grand River and 160 Sydenham River) with an average of 73.7 sequences per sample (range = 1 to 571 reads/sample); after BLASTing, one sequence returned a match for Johnny Darter (*Etheostoma nigrum*) and additional sequences matched to Black Redhorse (*Moxostoma duquesnei*), Creek Chub (*Semotilus atromaculatus*), Logperch (*Percina caprodes*), White Sucker (*Catostomus commersonii)*, and several matches to Round Goby. The species primers for Northern Madtom did not return any positive sequence reads. Only species with ≥ 3 eDNA sequences in a sample were kept for further spatial analysis; this included Ghost Shiner from the Silver Shiner primer set for three Grand River sites (G8, G29, G35), and Round Goby for one site in Grand River (G13); the rest were not used for further analysis. The species primers targeted a larger amplicon size (> 400 bp after library preparation) which resulted in lower amplification efficiency for NGS applications. The Northern Madtom primer set targeted a shorter amplicon size, however primer-template competition and sequencing bias was evident in this multiplex approach, and future studies should use primer sets targeting uniform, shorter amplicon sizes for eDNA and NGS protocols.

Community PS1 primer set: A total of 2 799 872 reads were returned for 342 (164 Grand River and 178 Sydenham River) samples with an average of 7527 sequences per sample (range = 1 to 52 556 reads/sample). After BLASTing against the custom fish COI sequence database, 970 578 sequences were returned where 77.0% matched Round Goby (746 868 sequences), 0.30% matched Silver Shiner (2805 sequences), 0.22% matched Eastern Sand Darter (2126 sequences) and 0.18% matched Northern Madtom (1739 sequences). The remaining 22.4% sequence reads (217 040 sequences) matched to 47 co-occurring native species. Grand River had a total of 353 267 returned eDNA sequence reads from 42 sites. Sydenham River had one

river control contaminated with Round Goby DNA, thus we excluded site S16 from further analysis, resulting in a total of 617 272 eDNA sequence reads from 43 sites.

Combined primer sets: Combining data from all five primer sets (species and community), eDNA was amplified for 44 of 67 fish species in Grand River at 42 of 43 sites, in a total of 158 samples out of 170 (53 bottom and 105 surface). In Sydenham River, eDNA was amplified for 44 of 67 species at 43 of 43 sites (note: site S16 removed) in a total of 174 samples out of 184 samples (1 river control, 69 bottom, and 104 surface) (Table 3.2). Round Goby was detected in 349 samples in total from both rivers; this corresponds to the majority of sampled sites. The number of Round Goby eDNA sequence reads per site varied widely, with a mean of 5663 sequences per site in the Grand River (range = 8 to 20 329 reads/site) and mean of 12 364 sequences per site in the Sydenham River (range = 3 to 51 507 reads/site). Round Goby was excluded from 1 Sydenham River site (S28) and 3 Grand River sites (G17, G18, and G24; Fig 1). Eastern Sand Darter was detected in 4 Grand River sites (G1, G9, G10, and G23) with a mean of 245 sequences per site (range = 61 to 683 reads/site), and 4 Sydenham River sites (S4, S29, S39, and S40) with a mean of 286 sequences per site (range = 187 to 506 reads/site). Silver Shiner was only detected in Grand River at 3 sites (G7, G10, and G40) with a mean of 935 eDNA sequences (range = 10 to 1044 reads/site). Northern Madtom was detected in 1 Grand River site (G6) in a surface sample with 50 eDNA sequence matches. Northern Madtom was also detected at 7 sites in Sydenham River (S4, S8, S14, S15, S21, S30, and S41) with a mean of 241 sequences (range = 4 to 1160 reads/site) (Fig. 3.2).

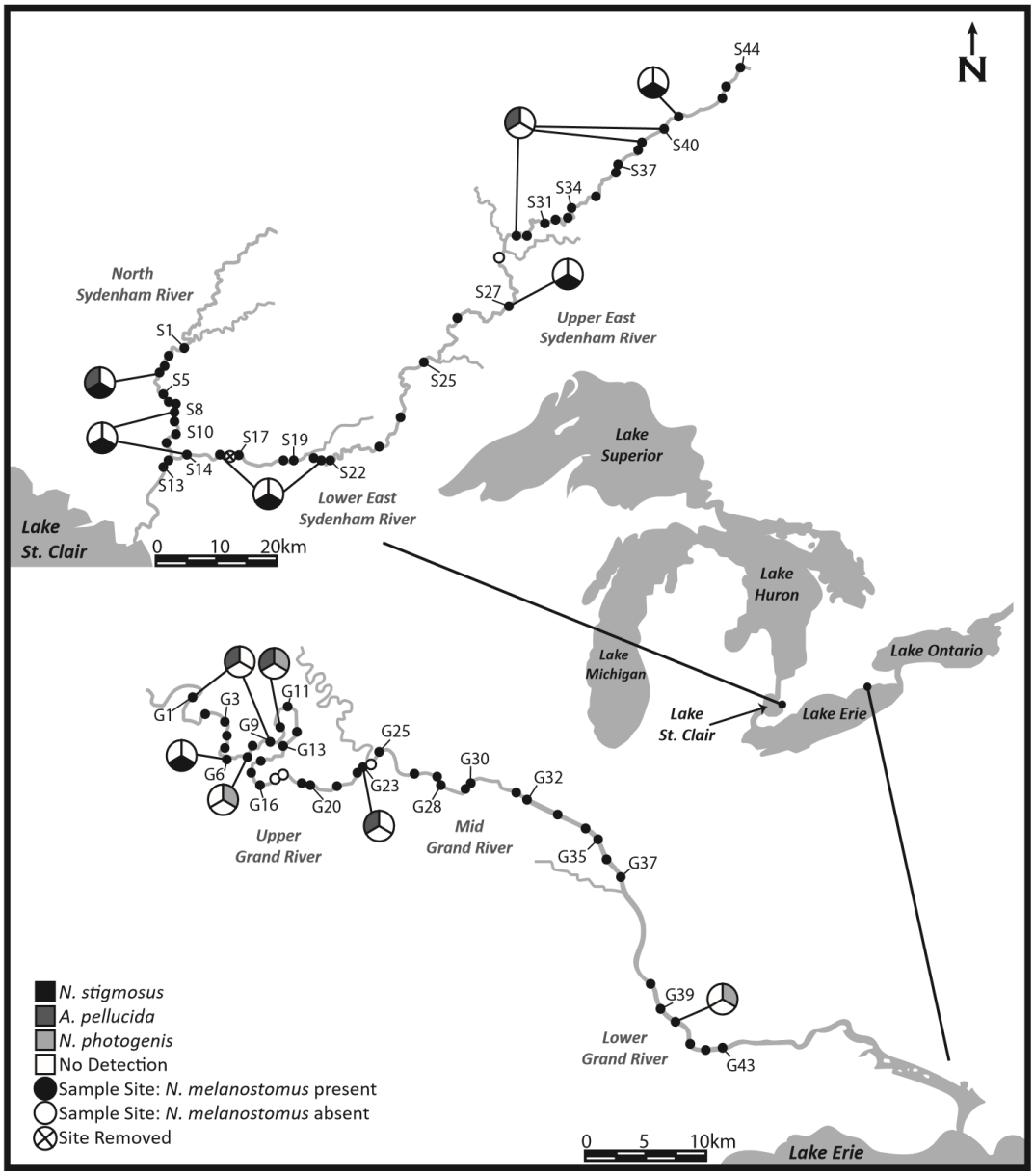

Figure 3.2. Map showing the spatial distribution of three species at risk (Northern Madtom *Noturus stigmosus*, Eastern Sand Darter *Ammocrypta pellucida*, Silver Shiner *Notropis photogenis*) and the invasive Round Goby (*Neogobius melanostomus*) in Sydenham River (44 sites) and Grand River (43 sites) in southern Ontario detected using environmental DNA.

Additionally, Eastern Sand Darter had higher sequence reads in surface samples than bottom samples, Northern Madtom had greater eDNA read count in surface samples, and Silver Shiner had greater eDNA read count in bottom samples (Fig. 3.3).

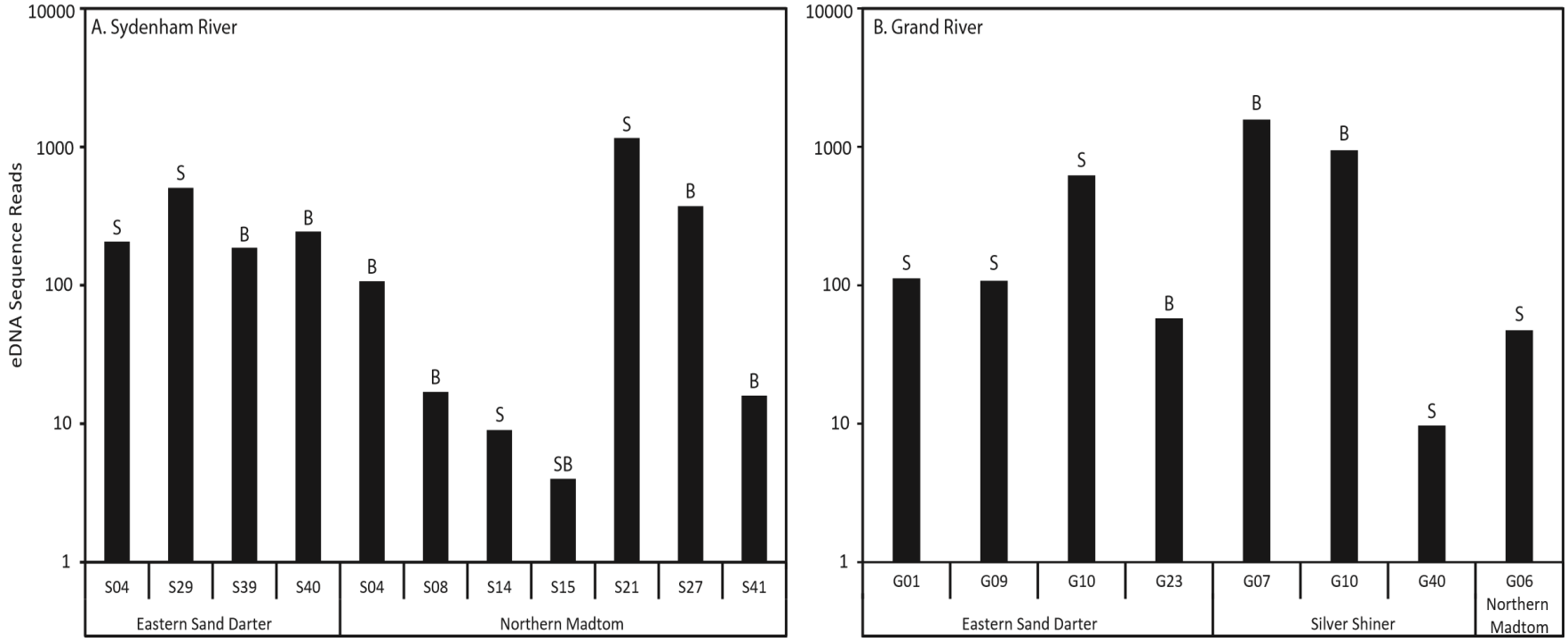

Figure 3.3. Number of eDNA sequence reads per site for positive detection of selected species at risk (Eastern Sand Darter (*Ammocrypta pellucida*), Northern Madtom (*Noturus stigmosus*), and Silver Shiner (*Notropis photogenis*)) in (A) Sydenham River and (B) Grand River. B = bottom sample, S = surface sample, SB = both samples.

Comparison with DFO Capture-Based Detection

Comparison of eDNA analysis and DFO conventional data was performed only on overlapping sample sites (18 sites in Sydenham River and 29 sites in Grand River). There was close agreement between the eDNA species identification and the capture-based species detection in Grand River (Fig. 3.4a), but results disagreed significantly in Sydenham River (Fig. 3.4b). Out of 67 species that were used in the custom river database, eDNA methods detected 40 of 67 (59.7%) species in Grand River whereas capture-based methods identified 47 of 67 (70.1%) species in overlapping sites. In Sydenham River, eDNA detected 27 of 67 (40.3%) species and capture-based methods identified 56 of 67 (83.6%) species in overlapping sites. Consequently, there was no statistical difference between eDNA and capture-based species detection for the Grand River (McNemar $\chi^2 = 1.79$, df = 1, $P = 0.18$), however there was a statistically significant difference in the detection success in Sydenham River, where conventional methods detected more species (McNemar $\chi^2 = 25.3$, df = 1, $P < 0.0001$). When analyzing the number of species detected by eDNA from all sampled sites, we detected 47 native species and our four target species (51 species total) from both rivers (Fig. 3.5)

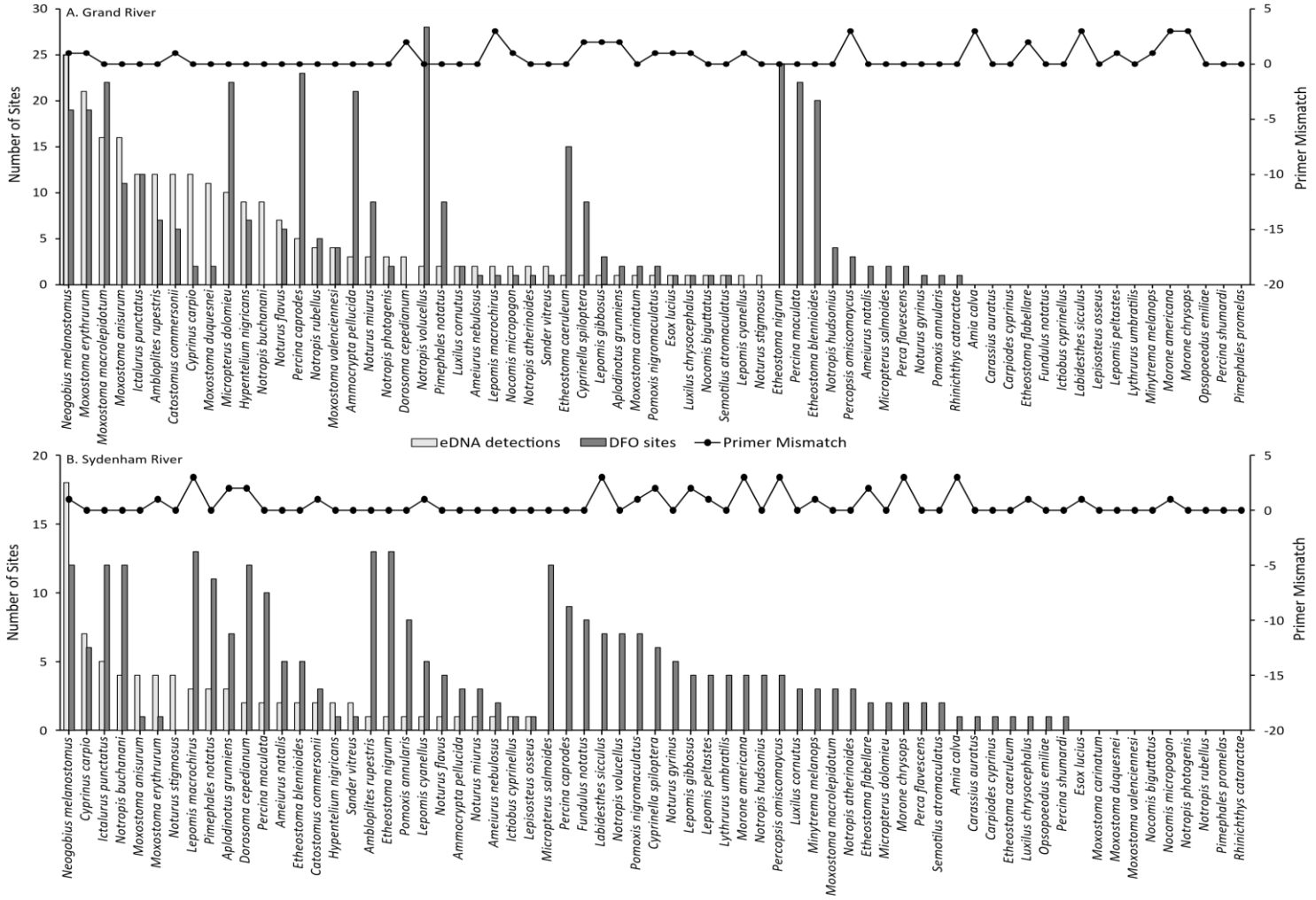

Figure 3.4. Histogram showing the number of sites each fish species was detected at using eDNA (dark grey) versus capture-based methods (light grey) from both (A) Grand River and (B) Sydenham River; only water sampling sites that overlapped with DFO capture sites were used in this analysis. Black line above the histogram shows the number of forward and reverse primer mismatches against known species COI sequences. Total number of species identified in the Grand River and Sydenham River by either eDNA, capture identification, or both was 67.

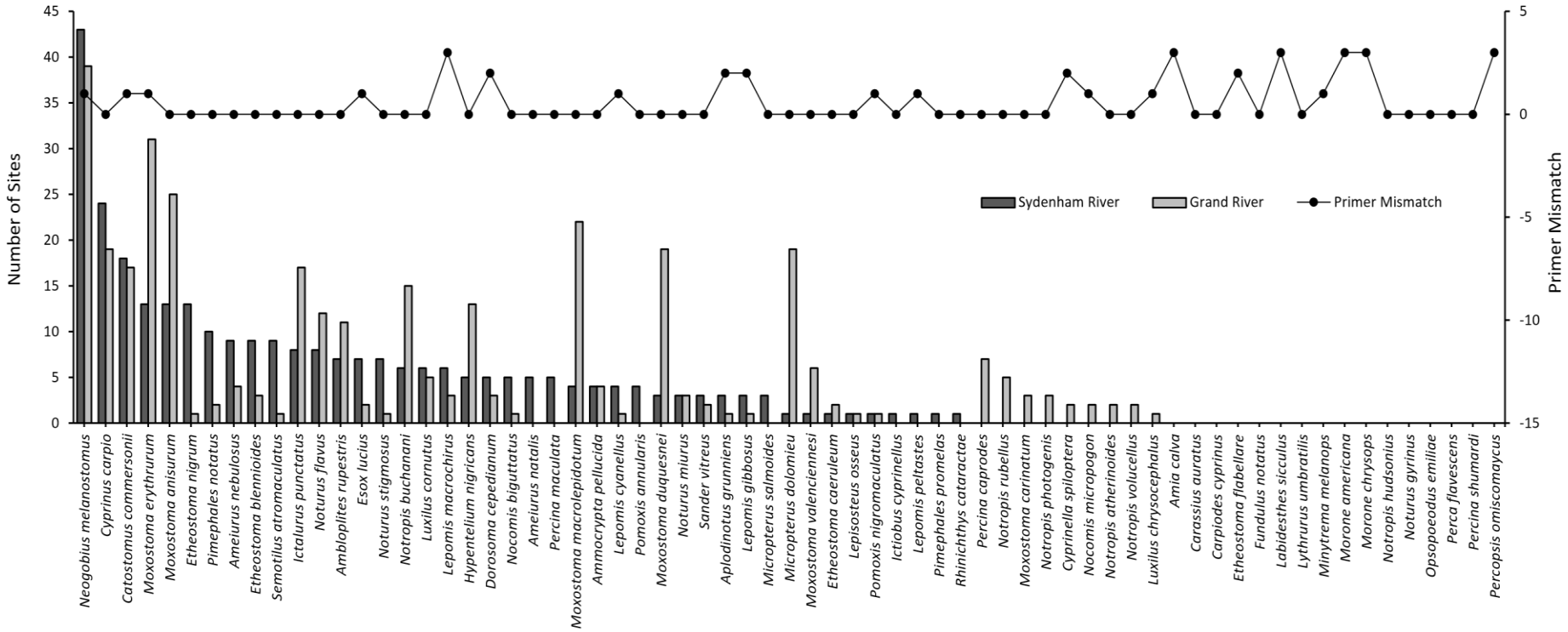

Figure 3.5. Histogram showing the number of sites a species (N = 67) was detected in using eDNA and next-generation sequencing analysis in Sydenham River (N = 43) and Grand River (N = 43). Positive detection at a site required ≥ 3 eDNA sequence matches per sample per site. Total of 43 species detected in Grand River, and 42 species in Sydenham River. The black line above the histogram shows the number of forward and reverse primer mismatches against the known species COI sequence. Number of species detected from both rivers using eDNA was 51.

Community and Target Species Co-Occurrence

A total of 1275 species-pair co-occurrences were analyzed using presence data for the 51 species detected using eDNA from both rivers. The majority of species co-occurrences were non-significantly different from that expected by chance (1139 pairs, $P > 0.05$), while 132 pairs had positive co-occurrence (occur together more often than expected) and 4 pairs had negative co-occurrence (occur together less often than expected) ($P < 0.05$, Fig. 3.6 and Table 3.4). For the four target species, Round Goby had positive co-occurrence with Common Carp ($P = 0.026$), Silver Shiner had positive co-occurrences with Black Redhorse (*Moxostoma duquesnei*, $P = 0.016$), Greater Redhorse (*Moxostoma valenciennesi*, $P = 0.017$) and Stonecat (*Noturus flavus*, $P = 0.011$), and Northern Madtom had positive co-occurrence with Walleye (*Sander vitreus*, $P = 0.0052$). Eastern Sand Darter did not depart from random expectations for any co-occurring species. Finally, the invasive Round Goby co-occurred with the species at risk at all of the identified sites.

Figure 3.6. Species co-occurrence matrix for 46 species with significant co-occurrences detected by the eDNA method. Positive interactions (dark grey squares) are species co-occurring more often than expected ($P < 0.05$), and negative interactions (black squares) are species that co-occur less often than expected ($P < 0.05$). Random co-occurrence (light grey squares) are co-occurrences that do not differ from random expectations ($P > 0.05$). In total, 1275 species pairs were analyzed, and 136 species-pair co-occurrences were significantly different from random expectations (Table 3.4). Squares with black borders represent significant co-occurrences with a target species. The matrix does not include species that showed only random expectation for co-occurrence with all other species.

Discussion

From both rivers, we detected 51 of 67 fishes which included all four of our target species using eDNA and NGS analysis. Although both capture-based identification and eDNA analysis detected Eastern Sand Darter in both rivers, sites did not coincide in both rivers. Eastern Sand Darter was detected mostly in lower East Sydenham River using conventional methods, but we detected high eDNA sequence read numbers (> 100) farther upstream in upper East Sydenham River and in North Sydenham River. The eDNA based distribution may be indicating movement of individuals upstream or perhaps the discovery of new populations. Capture-based methods detected Eastern Sand Darter in several sites throughout Grand River whereas eDNA detections were isolated to upper Grand River. Lack of Eastern Sand Darter eDNA detections downstream could also indicate that there are fewer individuals located downstream, resulting in weaker eDNA concentrations in downstream samples. Since eDNA has the potential for downstream migration in lotic systems, eDNA signals can reflect upstream individuals (Thomsen & Willerslev 2015); however, there is no mechanism for downstream eDNA sources to be detected upstream. Silver Shiner was detected only in Grand River using eDNA, which agrees with DFO capture data and documentation of its distribution (DFO 2013). Northern Madtom was detected in Sydenham River and returned 50 eDNA sequence matches at one Grand River site (G6). In both cases, the eDNA detections do not agree with DFO capture records and past reports of Northern Madtom presence. Although Northern Madtom have not been reported in the Sydenham River since 1975 (Edwards et al. 2012), the high number of eDNA sequence matches coupled with detection in a few independent sites (Fig. 3.3) suggests that there is a source of eDNA in Sydenham River, perhaps missed by conventional methods due to low abundance and cryptic behaviour of the Northern Madtom (COSEWIC 2002). Furthermore, our eDNA sequences had a 96%-99% identity match to Northern Madtom COI sequences from our

custom database, and only a 91% for Stonecat which co-occurs with our target species. An alternative possibility is that we detected residual eDNA dating to their known presence in the 1970's; however, such a long residence time for residual eDNA is highly unlikely, especially as we detected Northern Madtom DNA in surface water samples. While the detection of Northern Madtom at one Grand River site was based on 50 recovered sequence reads, the fact that it was at one isolated upstream site indicates it is likely a signal of a very few, isolated individuals. Northern Madtom have been reported in western Lake Erie (Manny et al. 2014), and our detection may have resulted from a few individuals moving into Grand River; however, it is very unlikely that Northern Madtom (or their eDNA) could have migrated upstream such a distance. Although eDNA sample contamination is a possibility, lab controls for this region of the Grand River did not produce amplification for any fish species (although there was no river control taken at this specific site). Previous work has shown that eDNA has a higher detection sensitivity, especially for rare or cryptic species, than conventional capture methods, and has extended the known range of a variety of species, including invertebrates (Tréguier et al. 2014), fish (Janosik & Johnston 2015; Sigsgaard et al. 2015) and amphibians (Pierson et al. 2016). For example, Sigsgaard et al. (2015) detected the European Weather Loach (*M. fossilis*) using eDNA at a site where it had not been detected by conventional methods for 17 years. Pierson et al. (2016) found that eDNA had 20X the detection probability for Patch-Nosed Salamander (*Urspelerpes brucei*) than traditional leaf litterbag surveys. Janosik and Johnston (2015) showed that traditional seining and netting identified the rare Slackwater Darter (*Etheostoma boschungi*) in 1 out of 49 sampled sites whereas eDNA identified them in 23 of the sites. Hence, while our eDNA detection of species at risk did not directly correspond to capture detections, the differences are likely attributable to the higher detection sensitivity of eDNA methods and the potential for target species movement between the time of the capture surveys and the

water sampling. Overall, our results highlight the potential for eDNA to be used alongside conventional methods, or as a stand-alone detection methodology for monitoring and mapping target species at risk.

The spread of aggressive invasive species such as the Round Goby can cause detrimental changes to local habitat and impact native biodiversity via competition and direct predation (Thompson & Simon 2014; Burkett & Jude 2015). Not surprisingly, a large portion (77.0%) of our eDNA sequence reads matched Round Goby DNA, with detections in 95% of all sampled sites in both rivers. The Round Goby is a known aggressive invader, with high-density capture-based detections in both the Sydenham River and Grand River since its secondary invasion of Great Lakes tributaries (Poos et al. 2010). eDNA analysis detected Round Goby in 9 Grand River and 5 Sydenham River sites where no individuals were captured using conventional methods. The additional Grand River sites were upstream of where past Round Goby captures occurred, indicating that they had moved upstream from the time conventional methods were used to when eDNA sampling occurred. The additional Sydenham River sites were also upstream from past capture records, however two extra sites with Round Goby eDNA detections were downstream of past capture data, signaling that downstream migration of Round Goby eDNA may have contributed to those detections.

Interestingly, sites in upper Grand and East Sydenham rivers had an overall higher number of detected fish species (≥ 10 species) including the three target species at risk (Fig. 3.2), implying that those areas may be localized hotspots for Great Lakes fishes. The use of appropriate, non-invasive management of Round Goby to limit their numbers while maintaining the relatively higher species richness in the upper reaches of these two large Great Lakes tributaries should be considered. For example, Round Goby pheromone traps designed to

attract only Round Goby would be ideal (Kornis et al. 2012b; Smith 2014). Curiously, the few sites that did not produce positive Round Goby eDNA detections had individuals captured. Thus Round Goby are present at those sites and the failure of our eDNA analysis to detect them is likely due to low eDNA quality/quantity in those samples. This is supported by the overall low number of eDNA sequence reads for all other species at those sites (range = 1 to 136 reads/species; Table 3.3). The risk of false negatives in eDNA methods can be costly when monitoring the spread of invasive species. In this study, eDNA had higher detection rates for the invasive Round Goby compared to conventional methods, and eDNA extended the Round Goby range farther upstream in both rivers. In sites where conventional methods had no Round Goby captured, neighbouring sites had few individuals captured. However, eDNA detected Round Goby in these sites demonstrating that negative detection rates are still higher in conventional methods, which is more detrimental for invasive species monitoring, and eDNA methods should be incorporated in early invasion studies.

The majority of species detected using eDNA co-occurred as expected based on random distributions. Overall, 127 species pairs showed positive co-occurrence and 4 species pairs showed negative co-occurrences. Rivers consist of a variety of habitats (i.e. patchy habitats) which can sustain a variety of species. The higher proportion of positive co-occurrences compared to negative co-occurrences is likely due to patchy habitats that are suitable to a majority of the native species. The few negative co-occurrences may represent highly divergent resource or habitat use between species pairs, or high resource competition. As an example, Gizzard Shad (*Dorosoma cepedianum*) and White Sucker have negative co-occurrence and both species feed on plankton (Whitehead 1985; Page & Burr 2011), except the White Sucker is more abundant in the two rivers and likely outcompetes the shad across several habitats.

Of the three target species at risk, only Silver Shiner and Northern Madtom exhibited non-random co-occurrence patterns with other native species (the Eastern Sand Darter was found to co-occur with all other species as expected based on random expectations). Silver Shiner positively co-occurred with Stonecat, Black Redhorse and Greater Redhorse. The co-occurrence patterns of these four species may reflect similarity in species habitat preference or resource use. There is little published on Stonecat biology in Ontario, however, it has been found in slow to fast moving riffles with rocky, gravel, and boulder substrate (Lane et al. 1996) which may coincide with Silver Shiner that also inhabit fast flowing riffles with rocky and boulder substrate (COSEWIC 2011). Likewise, Redhorse species are found inhabiting deep pools with gravel and boulder substrate (Bunt & Cooke 2001) perhaps driving its higher co-occurrence with the Silver Shiner. Northern Madtom was found to co-occur with Walleye more frequently than expected. Walleye is a piscivorous fish which have recently shifted its diet to include Round Goby (MacDougall et al. 2007; Reyjol et al. 2010), potentially reducing competition for Northern Madtom by reducing the Round Goby population in shared habitats. Characterizing co-occurring species with species of conservation concern can provide insight into potential species interactions (direct or indirect) that may be important for more effective conservation of species of interest. For the target invasive species, Round Goby was found to co-occur more often than expected with Common Carp, also an introduced species; however, identifying significant co-occurrences (i.e. non-random) with Round Goby is difficult to ascertain as they are broadly distributed in both rivers.

By using multiple PCR primers coupled with NGS for eDNA analyses, we not only detected our species of interest, but we also gathered distributional data for the fish community and significant species co-occurrences which may explain target species spatial distribution. Our eDNA based approach detected more than half of the native species known to be in the Grand

River, comparable to DFO capture efforts, and more than half of the native species in Sydenham River. The remaining native species not detected by eDNA methods was likely due to a combination of greater primer-template mismatches and low abundance (Fig. 3.5 and Table 3.3). eDNA metabarcoding is useful for whole community analyses, simultaneously monitoring ecosystem health by assessing species' distribution patterns (e.g. spread of known invaders), while monitoring for newly introduced species, unknown native species, and species at risk.

Conventional methods have very low detection rates when species are present in very low numbers leading to false negatives, but detection rate increases as the number of individuals increases when the species is abundant. However, for eDNA methods, detection rate also increases as target species abundance increases but the detection rate will plateau as eDNA will continue to detect the *presence* of the species regardless of how big the population grows. But, an important advantage of eDNA for species detection is its lower relative risk of false negatives for rare species due to its overall higher detection sensitivity when compared to conventional methods (Thomsen et al. 2011; Rees et al. 2015). Nevertheless, sampling season, number of replicate water samples (Ficetola et al. 2015), DNA extraction protocols (Renshaw et al. 2015; Takahara et al. 2015; Eichmiller et al. 2016), and primer design (Wilcox et al. 2013; Wilcox et al. 2014) influence the detection sensitivity of eDNA (Darling & Mahon 2011; Goldberg et al. 2015; Jerde & Mahon 2015; Rees et al. 2015). Although we expected to find a difference in eDNA detection of our target species between bottom and surface samples based on their known habitat use, we did not find any consistent pattern (Fig 3.3). This highlights the unpredictable nature of eDNA movement within the water column, and sampling protocols should include several sample replicates taken at various depths. Furthermore, in our comparison of overlapping eDNA and DFO capture sites, we found species which were captured but not detected using eDNA, especially in the Sydenham River (Fig. 3.4b). This may be due to

the high capture effort deployed in the Sydenham River coupled with the difference in sampling seasons, which can affect eDNA production and persistence. Fish were captured in the Grand River mostly using Missouri trawling and bag seining, whereas in Sydenham River, a range of diverse capture methods (e.g. trawling, mini fyke nets, backpacking, gill nets) were used, increasing the chance of detecting more species. Sampling season also likely affected our eDNA detections due to influence of fish biology and behaviour on eDNA production rates (Fukumoto et al. 2015; Spear et al. 2015). For example, Fukumoto et al. (2015) found greater eDNA detection frequency for the rare Japanese Giant Salamander (*Andrias japonicus*) when water samples were collected during the spawning season in September and December in the Katsura River basin, than in March and June. Spear et al. (2015) also had higher eDNA detection rates during the Eastern Hellbender (*C. alleganiensis*) breeding season. Therefore, since the majority of Great Lakes fishes spawn during spring to early summer (Lane et al. 1996), eDNA production rates and consequently, detection rates, were likely lower during mid to late October which was when Sydenham River samples were collected. To improve overall eDNA detection rates, multiple sampling trips (Bohmann et al. 2014; Boothroyd et al. 2016) and sampling during known breeding seasons can increase eDNA detection rates and thus reduce false negatives.

Despite approximately equal numbers of species detected in both rivers (43 species in Grand River, 42 species in Sydenham River), there was greater biodiversity variation among sites (beta diversity) in the Grand River (Table 3.3). The lower species richness among individual sites in the Sydenham River may be due to the early invasion of the Round Goby into the Sydenham River since its first detections in the St. Clair River in 1990 (Jude et al. 1992). Consequently, Round Goby colonized the geographically closer Sydenham River first, with early sightings of Round Goby in the Sydenham River in 1998 but not in the Grand River until 2005 (Poos et al.

2009), giving Round Goby seven extra years to disrupt the native community in the Sydenham River. As the Round Goby colonized the Sydenham River from the mouth, we would expect the impact of Round Goby on species communities to be highest in sites closer to the river mouth and decline as distance from the river mouth increased; however, no such pattern in the Grand River is expected as Round Goby colonized Grand River at multiple sites from the river mouth and further upstream (Bronnenhuber et al. 2011). To test these predictions, we compared site distances from the river mouth and site species number using presence data based on eDNA detections. There was a significant relationship between species numbers per site and increasing downstream distance in Sydenham River (Fig. 3.7; $R^2 = 0.50$, $P < 0.0001$), while the Grand River resulted in no significant relationship between distance and species numbers ($R^2 = 0.02$, $P = 0.44$). This implies that the invasion of Round Goby into the Sydenham River from Lake St. Clair may have contributed to the decrease in species richness in lower East and North Sydenham River. This pattern was not observed in Grand River, agreeing with the non-linear colonization of Round Goby in the Grand River (Bronnenhuber et al. 2011). Continued surveillance of native species community and Round Goby expansion in Grand and Sydenham rivers is critical to quantify the magnitude of the ecological impacts of the highly invasive Round Goby.

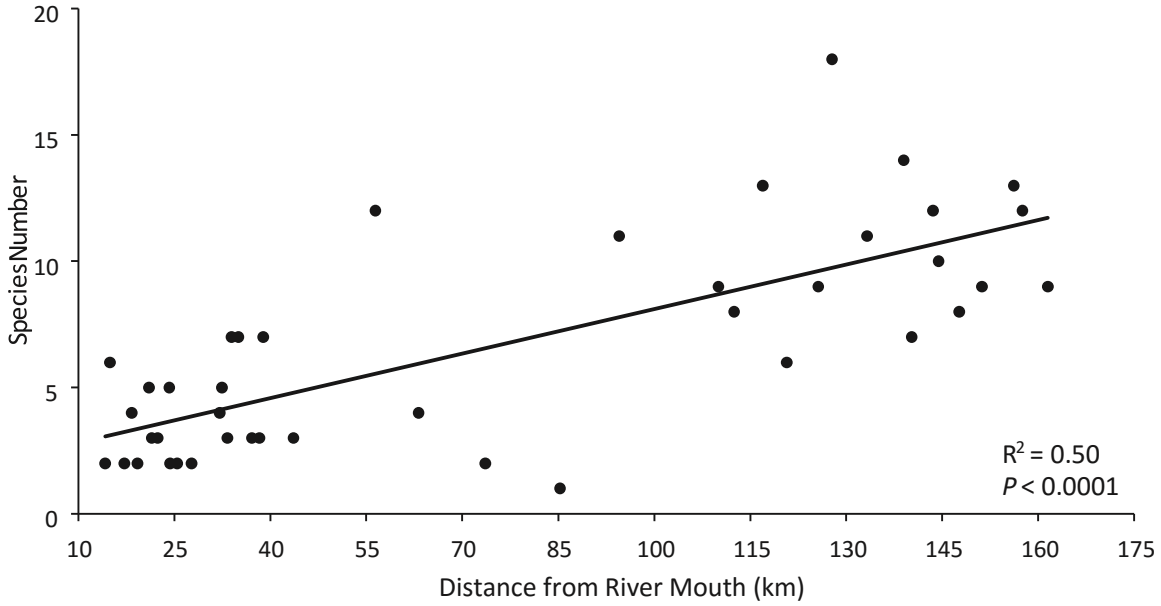

Figure 3.7. Correlation of site-specific species number (based on eDNA detections) and distance from the river mouth in the Sydenham River. Sites were excluded from this analysis if the number of Round Goby eDNA detections were below 3, or there was evidence for contamination. The significant correlation supports the hypothesis that the invasive Round Goby colonized the river from the river mouth and thus the impact of the Round Goby on species diversity would be higher at the river mouth, and decline with distance (proxy for time since colonization).

Conservation programs require information on the spatial distribution of species at risk in order to preserve critical habitat or develop spatial management plans for the target species. In the past, the distribution of fish species was determined using the physical capture of individuals, but those methods are logistically difficult, expensive, unintentionally harmful to target and co-occurring species, harmful to the ecosystem, and may exhibit low detection sensitivity for species that rare, cryptic, or inhabit difficult to sample areas. Our study demonstrates that a one-time sampling effort for water from two very large Great Lakes tributaries can successfully detect more than half of the known native community, including species at risk which were detected in several sites not identified by conventional methods. Moreover, our eDNA analysis detected the invasive Round Goby at more sites than the DFO capture program. eDNA analysis and next-generation sequencing also provide the opportunity

to passively assess community structure and thus determine important species co-occurrence or interactions, since eDNA samples contain template DNA from all species inhabiting the same environment. Community assessment identifies key species interactions and overall species distribution across sites. Such data identifies critical habitat (e.g. biodiversity hotspots), and can thus improve on current management efforts that rely solely on presence data for single target species. As eDNA methodology continues to be refined, future studies should employ eDNA analysis and NGS (less subject to false negatives) together with conventional methods (less subject to false positives), for large scale monitoring of whole community structure and important interspecific relationships with critical target species.

Table 3.3.

Fish species currently or historically known to inhabit the Grand or Sydenham rivers (N = 67 species) based on DFO catch data (DFO, unpubl. data). *Scientific name* (Common Name). * Indicates specific target species in this study. This species list was used to generate the COI sequence database for sequence BLAST analysis. Values for "E" are the number of eDNA sequence matches, and values for "C" are number of live individuals captured using Missouri Trawl, bag seines, backpacking, or various netting methods (i.e. gill nets, trap nets). Blank cells indicate no detection or captured individuals. Site S16 removed due to eDNA contamination. Shaded sites were sampled using conventional methods and eDNA. G = Grand River sites, S = Sydenham River sites.

Sites	*Neogobius melanostomus* (Round Goby)		*Ammocrypta pellucida* (Eastern Sand Darter)		*Notropis photogenis* (Silver Shiner)		*Noturus stigmosus* (Northern Madtom)		*Ambloplites rupestris* (Rock Bass)		*Ameiurus natalis* (Yellow Bullhead)		*Ameiurus nebulosus* (Brown Bullhead)		*Amia calva* (Bowfin)		*Aplodinotus grunniens* (Freshwater Drum)		*Carassius auratus* (Goldfish)		*Carpiodes Cyprinus* (Quillback)	
	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C
G1	169		120			18																
G2	289																					
G3	5075									2												
G4	5781												106									
G5	1686								6	4							83					
G6	3508						50		15													
G7	11411			139	1751				1037									139				
G8	17899								206													
G9	1425		115																			
G10	1872		683	35	1044	2			652								1	35				
G11	5022																					
G12	1105			159					416									159				
G13	11933	3		128					65	2				1				128				
G14	20329	1		131					166				37					131				
G15				54														54				
G16	89																					
G17		6		64														64				
G18	1			164														164				
G19	2																					
G20	231	6		125														125				
G21	1415	131		2						21			31					2				
G22	399																					
G23	2864	190	61	72								2						72				
G24	1	61		49														49				
G25	4273	297		17						1								17				
G26	16870																					
G27	5695	1106		2					203									2				
G28	1646																					
G29	18598	386							1678													
G30	6921	998		6														6				

G31	3241	1606								2													
G32	15515	752							1			1											
G33	1213																						
G34	2279												1463										
G35	8881	77											1										
G36	1912	457		2														2					
G37	7116																						
G38	12509	163		25						1								25					
G39	7457	934		47					53									47					
G40	1350	593		32	10													32					
G41	2387								24														
G42	8	1221		1														1					
G43	4815																						
S1	14021										19						55						
S2	25567	3								1	39	2											
S3	8523	10										1											
S4	12919	46	207				107			2		4		2		1				1			
S5	12370																						
S6	788												1										
S7	20639																						
S8	31897	54					17			9		1	34	1									
S9	12238																1						
S10	11446																						1
S11	23711																						
S12	6725		2																				
S13	9573	35								17													
S14	12422	123					9																
S15	6117						4																
S17	190	47								2													
S18	17839	85							1	3							9						
S19	3995	77								1							76						
S20	3498	178		46						6								46					
S21	2640	38					1160			1													
S22	15583	32		15					296	40								15					
S23	29991											1											
S24	24309	10		39						9								39					
S25	687									3													
S26	4																						
S27	15316						374		611														
S28	2																						
S29	29950		506																				
S30	2540						2		3														
S31	10676																						

S32	7701																					
S33	3218																					
S34	1012								39		65											
S35	19920												146									
S36	3577								92		161											
S37	6533												162									
S38	1095								18													
S39	4199		187								16											
S40	6299		245																			
S41	11479						16															
S42	51507																					
S43	25233								887													
S44	23696												280									

Table S1 Continued. Part 2 of 6

Sites	*Catostomus commersonii* (White Sucker)		*Cyprinella spiloptera* (Spotfin Shiner)		*Cyprinus carpio* (Common Carp)		*Dorosoma cepedianum* (Gizzard Shad)		*Esox lucius* (Northern Pike)		*Etheostoma blennioides* (Greenside Darter)		*Etheostoma caeruleum* (Rainbow Darter)		*Etheostoma flabellare* (Fantail Darter)		*Etheostoma nigrum* (Johnny Darter)		*Fundulus notatus* (Blackstripe Topminnow)		*Hypentelium nigricans* (Northern Hogsucker)		*Ictalurus punctatus* (Channel Catfish)	
	E	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**
G1		9		6								15	1	20				2				11		
G2	2				31																			
G3							115					25		1										
G4	25													5									136	
G5					61		20					104		200				671					20	
G6	3											10		33				17						2
G7	943		2									6		27				7			3821	5	831	
G8	1076			2	622							63		1							1237		1	
G9					63								1											
G10	199			7	358				10			37		3							342		44	
G11	320				214						279		1										767	
G12	379			1	411							47		5				61			272	5		
G13	112				15							13		4				107			103	3		
G14			12	1	1980	1						15					1	80			800			
G15	33			7								7						2				1		
G16					873																51		1	
G17				2								26		15				87				1		
G18																		98				1	16	
G19													1								1			
G20					1							4						56						1
G21				5	18							9		4				109						
G22																								
G23		1			63							5		1				141			90		36	3

G24		1																17						1
G25	118																	60					36	3
G26																							812	
G27	1				7													21					161	1
G28																								
G29			1																				86	
G30		1																8			1		478	
G31	21	2										1	10					9						
G32	1	1								1								10			203			
G33	13				8																44			
G34	1				28						1907										37		852	
G35					1131		67					16		1				1					33	1
G36	43				1372	1			3			28									2110		966	7
G37			6		73												17						1308	
G38				38	1									3									956	3
G39	126				12							1						10			2			10
G40	114													1				6						1
G41	177																							
G42	1											1						1						71
G43	63										55		3				1				28			
S1						1	84																104	16
S2					263	1		429										6		12				72
S3	1				1		51	6										13		44				46
S4						13	1	130										8		11				184
S5							107																	
S6					1				3															
S7							219																	
S8				1				8										8		23				
S9							1																4	
S10						1																	1	
S11						2																		
S12	2				3		622				1						1							
S13								1										1						1
S14	1							1															66	9
S15	7				878						1						7						1	
S17	2			1	11			5	1		10			1			2	6		33	1		3	
S18					20	2												1					5	2
S19	129				104													1						1
S20	1				375			1				3						17						5
S21	1																1							
S22	1			45				13				13						11		25				52
S23	251												7								416			
S24		1		113	45			29				50				5		97		2	96			8

S25		1		8				2			1	1280				7		110		1		1		91
S26																								
S27	368				91								2											
S28																								
S29					146																			
S30	315				334												545				6			
S31	319	1		70	713						367	55					460	71			55		1197	
S32					7						42												43	
S33	52				216				11		228						418							
S34	1280		1		1045				2		144						459						30	
S35	1850				568				24															
S36	510				1371				30		104						81							
S37	128				1571				17								213							
S38	1138				60						1						242							
S39	515				1523						74						148							
S40	136								7		42						290							
S41	803				642												998							
S42	2155				2770				97		25						751				673			
S43	2545				671																			
S44	2187				1487												2971							

Table S1 Continued. Part 3 of 6

Sites	*Ictiobus cyprinellus* (Bigmouth Buffalo)		*Labidesthes sicculus* (Brook Silverside)		*Lepisosteus osseus* (Longnose Gar)		*Lepomis cyanellus* (Green Sunfish)		*Lepomis gibbosus* (Pumpkinseed)		*Lepomis macrochirus* (Bluegill)		*Lepomis peltastes* (Northern Sunfish)		*Luxilus chrysocephalus* (Striped Shiner)		*Luxilus cornutus* (Common Shiner)		*Lythrurus umbratilis* (Redfin Shiner)		*Micropterus dolomieu* (Smallmouth Bass)		*Micropterus salmoides* (Largemouth Bass)	
	E	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**
G1										2		4				416		162						2
G2																					184	2		
G3																						2		
G4											58													
G5																						21		
G6																								
G7																	3	1			768	4		
G8																	1				504	2		
G9																					725			
G10																	1				1825	1		
G11																	1319				742			
G12											82						259				3	3		
G13									32	1					5						359	6		1
G14																						3		
G15																					53	3		
G16																	131							

G17																						7		
G18																	1							
G19																	117							
G20																						1		
G21																						11		
G22																								
G23											6											2		
G24										1												6		
G25																					33			
G26																								
G27																						2		
G28																					398			
G29																					306	5		
G30																					205	2		
G31																								
G32																								
G33																					121			
G34					118																421			
G35							628															19		
G36																					166	8		
G37																					46			
G38																						2		
G39																					2	1		
G40																								
G41																					18			
G42																								
G43																					90			
S1							234					1												
S2	103			1								7					1							2
S3				2						2	59	24												2
S4		8						3		1		22						1						18
S5																								
S6																								
S7																								
S8				9				1				63												4
S9																	1							
S10											13	3												
S11											75													
S12									4												1			
S13												18												2
S14												40												1
S15																								
S17				3				1		6		16												12

S18				1	6					1		8		1											
S19												1													2
S20												13		1											
S21																									1
S22				35		1	1					7		3						4		7			3
S23																					1211				
S24				5										9				13		21					7
S25																17		16		6		1			2
S26																	1								
S27																									
S28																									
S29																								127	
S30																									
S31																				1	1				
S32																									
S33																									
S34							18		38																
S35											57						84							14	
S36									186																
S37																									
S38							4						13											25	
S39																	75								
S40																									
S41																	247								
S42											780						4662								
S43							505				98						2710							2	
S44																	1277								

Table S1 Continued. Part 4 of 6

Sites	*Minytrema melanops* (Spotted Sucker)		*Morone americana* (White Perch)		*Morone chrysops* (White Bass)		*Moxostoma anisurum* (Silver Redhorse)		*Moxostoma carinatum* (River Redhorse)		*Moxostoma duquesnei* (Black Redhorse)		*Moxostoma erythrurum* (Golden Redhorse)		*Moxostoma macrolepidotum* (Shorthead Redhorse)		*Moxostoma valenciennesi* (Greater Redhorse)		*Nocomis biguttatus* (Hornyhead Chub)		*Nocomis micropogon* (River Chub)	
	E	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**
G1							2						10	1		2				12		6
G2							209				15		78		1							
G3													44									
G4													27									
G5							1							1		1	2					
G6							7						73	2	6							
G7							1001				1821		896		2913	7	845		9		9	
G8							235				1312		283	1	544		4					
G9							324				9		580		3							

G10							2317			1	944		2448		1040	4	806					
G11							376				1174		738		558		22		1			
G12							152				34		663	3	249	4	1					
G13							5	205			340	1	112	10		4	1		6			
G14							2635	1			863		5988	1	710	5						
G15							1						42		113	2		1				
G16							50				106		384		161							
G17														3		3						
G18								1					37	3								
G19							47				59				2							
G20								6			10		26	2	11	1						
G21												1		9		1					21	
G22													7									
G23							41				1		137	1	98							
G24							136	1			14		87	5		1						
G25							49	4					16	18		6			21			
G26							503						106									
G27								1					53		52	6		1				
G28							276						1									
G29							30							1	1639	5		1				
G30							719	1						3		1						
G31							192	10						1	147							
G32								1					2		11							
G33											9		20				1675					
G34							2039		73		33		1843		5563						1	
G35							101		3				117		33	1	1121					
G36							4280				58		3794		3073	1						
G37							1027		279		30		1027		426							
G38							2						6		40	1						
G39							30				163		57	4		1		2				
G40											25			13		2						
G41																						
G42								1					16			2		4				
G43							1								19							
S1																						
S2						4																
S3				5																		
S4		1		6				1								4						
S5																						
S6													1									
S7																						
S8		1																				
S9																						

S10																								
S11																								
S12							4													1				
S13		1		10																				
S14				2		3											1							
S15																								
S17													1											
S18																								
S19													27											
S20							12													1				
S21													11											
S22							30											36						
S23							641				1029		364		755									
S24							198									2								
S25													29											
S26																								
S27							43				82		25		25									
S28																								
S29							335				202		165		181									
S30							99						73											
S31							266				1		310	2										
S32													91											
S33													92											
S34							35						233		37									
S35													1							182				
S36							325						122							108				
S37													1											
S38							158																	
S39							1								1					140				
S40													243											
S41							159								1									
S42							1													28				
S43																				1880				
S44																								

Table S1 Continued. Part 5 of 6

Sites	*Notropis atherinoides* (Emerald Shiner)		*Notropis buchanani* (Ghost Shiner)		*Notropis hudsonius* (Spottail Shiner)		*Notropis rubellus* (Rosyface Shiner)		*Notropis volucellus* (Mimic Shiner)		*Noturus flavus* (Stonecat)		*Noturus gyrinus* (Tadpole Madtom)		*Noturus miurus* (Brindled Madtom)		*Opsopoeodus emiliae* (Pugnose Minnow)		*Perca flavescens* (Yellow Perch)		*Percina caprodes* (Logperch)		*Percina maculata* (Blackside Darter)	
	E	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**
G1								793		463					98							2		
G2			155																					

G3										1															1
G4																									
G5										3		1											1		20
G6										1															
G7	11	10	1143		1751		1336		1	48	809	1										2			2
G8			2797					2			58			1								1			
G9			202								141														
G10					1044			2		9	178										1				
G11			3								5														
G12			1							272															21
G13			144							379	34					8				1		9			74
G14			931				1245			250		1									425	5			19
G15								1		240												3			2
G16			316																						
G17										341						6						5			12
G18			195							136	4					1									9
G19							49																		
G20						1				273		1				6						4			16
G21										898						2						14			75
G22																									
G23										222						4					6	2			15
G24										378						3				1		4			40
G25										108															18
G26			303																						
G27									292	145											137	4			14
G28			743																						
G29			787							98												10			
G30										262												13			17
G31										58	45				50	1						7			7
G32										5						1						5			11
G33			474																						
G34			1867																		646				
G35			246						4	93		1									339	48			1
G36	1364						307			79		1			120						575	23			1
G37																					396				
G38								6		4	7											36			
G39						23				60	70											63			1
G40					10	73				449	10											39			
G41																									
G42						34				110	25											13			1
G43															1										
S1			381	326										1											
S2			30	750						4				14											4

S3				501										25						1					
S4				1305						10				3								2		5	
S5																									
S6																									
S7																									
S8				108		3								9								1		6	
S9																									
S10																									
S11			331																						
S12			5																						
S13				1779		8														1		3		3	
S14				1826		2																5			
S15																									
S17		2								5								5					20		
S18				2057		4																		2	
S19				614											88							1			
S20				650						1		1				8						3		35	
S21				19																		1	1	3	
S22		5		151						75	160					6						30		34	
S23																									
S24		1								218		3				10						24		73	
S25										137		47										2		90	
S26											27														
S27															5										
S28																									
S29															224										
S30			141								35												1		
S31												1											941		
S32											3														
S33			203								120												243		
S34																							283		
S35											74														
S36																									
S37																							1		
S38											1														
S39																									
S40																									
S41																									
S42											3				1								34		
S43											78														
S44																									

Table S1 Continued. Part 6 of 6

Sites	*Percina shumardi* (River Darter)		*Percopsis omiscomaycus* (Trout-Perch)		*Pimephales notatus* (Bluntnose Minnow)		*Pimephales promelas* (Fathead Minnow)		*Poxomis annularis* (White Crappie)		*Poxomis nigromaculatus* (Black Crappie)		*Rhinichthys cataractae* (Longnose Dace)		*Sander vitreus* (Walleye)		*Semotilus atromaculatus* (Creek Chub)	
	E	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**
G1												1		28				3
G2																		
G3																		
G4																		
G5						2					79							
G6															21			
G7																		
G8																		
G9																		
G10					1													
G11																		
G12						1												
G13						16												
G14															507			
G15																		
G16																		
G17																		
G18					68													
G19																		
G20																		
G21						7												
G22																		
G23																	2	
G24						3												
G25																		
G26																		
G27																		
G28																		
G29																		
G30																		
G31						13												
G32																		
G33																		
G34																		
G35					920													
G36																		
G37																		

G38																		
G39				148		2												
G40				127		8											94	
G41																		
G42				60		6				2		1				1		
G43																		
S1					145					2								
S2					721				162	12		18						
S3						4				8		10						
S4						5				20		21						
S5																		
S6																		
S7																		
S8						10				22		60						
S9					8													
S10																		
S11					1													
S12					2												1	
S13						1				38								
S14		2		1						18		2			1059			
S15																	2	
S17						34									2			
S18				7		3				1		1						
S19				1											1320			
S20				3		12											1	
S21																		
S22						67						1						
S23													348					
S24						133										1		
S25						255												6
S26																		
S27																		
S28																		
S29																		
S30																		
S31					273	9												3
S32					470													
S33																		
S34					295										1		58	
S35																	193	
S36					175										1			
S37											201							
S38					11		225										70	

S39																	6	
S40																	197	
S41															36		191	
S42																	1215	
S43					1379												92	
S44					1480												1894	

Table 3.4.

Statistically significant species co-occurrences ($P < 0.05$) which includes the two target species at risk (Northern Madtom - *Noturus stigmosus*, and Silver Shiner - *Notropis photogenis*), one target invasive species (Round Goby - *Neogobius melanostomus*), and 43 natives (Target species at risk Eastern Sand Darter – *Ammocrypta pellucida* and 21 natives did not produce significant co-occurrence, i.e. had random co-occurrence $P > 0.05$). Analysis was done using R Studio v. 0.99.892 (RStudio Team 2015) and R package "cooccur" (Griffith et al. 2016). [a] is the probability that the species pair co-occurs less often than observed (negative co-occurrence) and [b] is the probability that the species pair is likely to co-occur more often than observed.

N Sites Sp. 1 Occurs	N Sites Sp. 2 Occurs	N Sites Sp. 1 and Sp. 2 Co-Occur	Probability of Sp. 1 and Sp. 2 Co-Occurrence	Expected N Sites with Sp. 1 and Sp. 2	[a]p_lt	[b]p_gt	Sp. 1 Name	Sp. 2 Name
19	35	14	0.092	7.8	1.000	***0.001***	*Ambloplites rupestris*	*Catostomus commersonii*
19	43	14	0.113	9.6	0.995	***0.020***	*Ambloplites rupestris*	*Cyprinus carpio*
19	4	3	0.011	0.9	0.998	***0.034***	*Ambloplites rupestris*	*Lepomis gibbosus*
19	38	15	0.1	8.5	1.000	***0.001***	*Ambloplites rupestris*	*Moxostoma anisurum*
19	26	10	0.068	5.8	0.995	***0.021***	*Ambloplites rupestris*	*Moxostoma macrolepidotum*
19	20	10	0.053	4.5	1.000	***0.002***	*Ambloplites rupestris*	*Noturus flavus*
5	12	3	0.008	0.7	0.999	***0.019***	*Ameiurus natalis*	*Etheostoma blennioides*
5	14	3	0.01	0.8	0.998	***0.030***	*Ameiurus natalis*	*Etheostoma nigrum*
5	5	2	0.003	0.3	0.999	***0.026***	*Ameiurus natalis*	*Lepomis cyanellus*
5	4	2	0.003	0.2	1.000	***0.016***	*Ameiurus natalis*	*Lepomis gibbosus*
5	6	2	0.004	0.4	0.998	***0.038***	*Ameiurus natalis*	*Nocomis biguttatus*
5	12	4	0.008	0.7	1.000	***0.001***	*Ameiurus natalis*	*Pimephales notatus*
4	8	2	0.004	0.4	0.998	***0.043***	*Aplodinotus grunniens*	*Dorosoma cepedianum*
35	43	24	0.208	17.7	0.999	***0.005***	*Catostomus commersonii*	*Cyprinus carpio*
35	8	0	0.039	3.3	***0.011***	1.000	*Catostomus commersonii*	*Dorosoma cepedianum*
35	9	8	0.044	3.7	1.000	***0.003***	*Catostomus commersonii*	*Esox lucius*
35	12	9	0.058	4.9	0.998	***0.012***	*Catostomus commersonii*	*Etheostoma blennioides*
35	14	13	0.068	5.8	1.000	***0.000***	*Catostomus commersonii*	*Etheostoma nigrum*
35	18	12	0.087	7.4	0.997	***0.014***	*Catostomus commersonii*	*Hypentelium nigricans*

35	11	9	0.053	4.5	1.000	***0.005***	*Catostomus commersonii*	*Luxilus cornutus*
35	20	13	0.097	8.2	0.997	***0.014***	*Catostomus commersonii*	*Micropterus dolomieu*
35	7	6	0.034	2.9	0.999	***0.018***	*Catostomus commersonii*	*Moxostoma valenciennesi*
35	6	6	0.029	2.5	1.000	***0.004***	*Catostomus commersonii*	*Nocomis biguttatus*
35	20	17	0.097	8.2	1.000	***0.000***	*Catostomus commersonii*	*Noturus flavus*
35	10	10	0.048	4.1	1.000	***0.000***	*Catostomus commersonii*	*Semotilus atromaculatus*
2	7	2	0.002	0.2	1.000	***0.006***	*Cyprinella spiloptera*	*Percina caprodes*
43	12	10	0.071	6.1	0.998	***0.015***	*Cyprinus carpio*	*Etheostoma blennioides*
43	14	13	0.083	7.1	1.000	***0.000***	*Cyprinus carpio*	*Etheostoma nigrum*
43	18	14	0.107	9.1	0.998	***0.009***	*Cyprinus carpio*	*Hypentelium nigricans*
43	11	9	0.065	5.6	0.996	***0.027***	*Cyprinus carpio*	*Luxilus cornutus*
43	38	26	0.226	19.2	0.999	***0.003***	*Cyprinus carpio*	*Moxostoma anisurum*
43	22	16	0.131	11.1	0.997	***0.014***	*Cyprinus carpio*	*Moxostoma duquesnii*
43	80	43	0.476	40.5	1.000	***0.026***	*Cyprinus carpio*	*Neogobius melanostomus*
43	6	6	0.036	3	1.000	***0.014***	*Cyprinus carpio*	*Nocomis biguttatus*
43	21	15	0.125	10.6	0.994	***0.025***	*Cyprinus carpio*	*Notropis buchanani*
43	7	7	0.042	3.5	1.000	***0.007***	*Cyprinus carpio*	*Percina caprodes*
43	5	5	0.03	2.5	1.000	***0.029***	*Cyprinus carpio*	*Percina maculata*
43	10	8	0.06	5.1	0.992	***0.048***	*Cyprinus carpio*	*Semotilus atromaculatus*
9	12	5	0.015	1.3	1.000	***0.002***	*Esox lucius*	*Etheostoma blennioides*
9	14	5	0.017	1.5	1.000	***0.005***	*Esox lucius*	*Etheostoma nigrum*
9	6	3	0.007	0.6	0.999	***0.014***	*Esox lucius*	*Nocomis biguttatus*
12	14	7	0.023	2	1.000	***0.000***	*Etheostoma blennioides*	*Etheostoma nigrum*
12	6	3	0.01	0.8	0.997	***0.034***	*Etheostoma blennioides*	*Nocomis biguttatus*
12	20	6	0.033	2.8	0.994	***0.030***	*Etheostoma blennioides*	*Noturus flavus*
12	5	5	0.008	0.7	1.000	***0.000***	*Etheostoma blennioides*	*Percina maculata*
12	10	4	0.017	1.4	0.996	***0.031***	*Etheostoma blennioides*	*Semotilus atromaculatus*
3	26	3	0.011	0.9	1.000	***0.026***	*Etheostoma caeruleum*	*Moxostoma macrolepidotum*
3	1	1	0	0	1.000	***0.035***	*Etheostoma caeruleum*	*Rhinichthys cataractae*
14	5	4	0.01	0.8	1.000	***0.002***	*Etheostoma nigrum*	*Percina maculata*
14	12	5	0.023	2	0.996	***0.024***	*Etheostoma nigrum*	*Pimephales notatus*
14	10	7	0.019	1.6	1.000	***0.000***	*Etheostoma nigrum*	*Semotilus atromaculatus*
18	20	10	0.05	4.2	1.000	***0.001***	*Hypentelium nigricans*	*Micropterus dolomieu*
18	38	14	0.095	8	1.000	***0.002***	*Hypentelium nigricans*	*Moxostoma anisurum*
18	22	11	0.055	4.7	1.000	***0.000***	*Hypentelium nigricans*	*Moxostoma duquesnii*
18	44	14	0.11	9.3	0.998	***0.012***	*Hypentelium nigricans*	*Moxostoma erythrurum*
18	26	12	0.065	5.5	1.000	***0.000***	*Hypentelium nigricans*	*Moxostoma macrolepidotum*
18	7	6	0.017	1.5	1.000	***0.000***	*Hypentelium nigricans*	*Moxostoma valenciennesi*
18	2	2	0.005	0.4	1.000	***0.043***	*Hypentelium nigricans*	*Notropis atherinoides*

18	21	8	0.052	4.4	0.992	***0.034***	*Hypentelium nigricans*	*Notropis buchanani*
18	20	8	0.05	4.2	0.995	***0.024***	*Hypentelium nigricans*	*Noturus flavus*
18	7	4	0.017	1.5	0.996	***0.034***	*Hypentelium nigricans*	*Percina caprodes*
25	3	3	0.01	0.9	1.000	***0.023***	*Ictalurus punctatus*	*Moxostoma carinatum*
25	44	17	0.152	12.9	0.986	***0.044***	*Ictalurus punctatus*	*Moxostoma erythrurum*
25	26	12	0.09	7.6	0.993	***0.025***	*Ictalurus punctatus*	*Moxostoma macrolepidotum*
25	7	5	0.024	2.1	0.998	***0.021***	*Ictalurus punctatus*	*Moxostoma valenciennesi*
25	21	10	0.073	6.2	0.990	***0.036***	*Ictalurus punctatus*	*Notropis buchanani*
25	7	6	0.024	2.1	1.000	***0.002***	*Ictalurus punctatus*	*Percina caprodes*
25	12	7	0.042	3.5	0.995	***0.025***	*Ictalurus punctatus*	*Pimephales notatus*
1	4	1	0.001	0	1.000	***0.047***	*Ictiobus cyprinellus*	*Pomoxis annularis*
5	12	5	0.008	0.7	1.000	***0.000***	*Lepomis cyanellus*	*Pimephales notatus*
5	10	3	0.007	0.6	1.000	***0.011***	*Lepomis cyanellus*	*Semotilus atromaculatus*
4	1	1	0.001	0	1.000	***0.047***	*Lepomis gibbosus*	*Luxilus chrysocephalus*
4	38	4	0.021	1.8	1.000	***0.036***	*Lepomis gibbosus*	*Moxostoma anisurum*
4	6	2	0.003	0.3	0.999	***0.024***	*Lepomis gibbosus*	*Nocomis biguttatus*
4	21	3	0.012	1	0.997	***0.045***	*Lepomis gibbosus*	*Notropis buchanani*
4	20	3	0.011	0.9	0.998	***0.039***	*Lepomis gibbosus*	*Noturus flavus*
9	11	4	0.014	1.2	0.999	***0.014***	*Lepomis macrochirus*	*Luxilus cornutus*
9	6	3	0.007	0.6	0.999	***0.014***	*Lepomis macrochirus*	*Nocomis biguttatus*
9	4	3	0.005	0.4	1.000	***0.003***	*Lepomis macrochirus*	*Pomoxis annularis*
1	3	1	0	0	1.000	***0.035***	*Lepomis peltastes*	*Micropterus salmoides*
1	1	1	0	0	1.000	***0.012***	*Lepomis peltastes*	*Pimephales promelas*
11	6	4	0.009	0.8	1.000	***0.002***	*Luxilus cornutus*	*Nocomis biguttatus*
11	4	3	0.006	0.5	1.000	***0.006***	*Luxilus cornutus*	*Pomoxis annularis*
11	10	6	0.015	1.3	1.000	***0.000***	*Luxilus cornutus*	*Semotilus atromaculatus*
20	38	16	0.105	8.9	1.000	***0.000***	*Micropterus dolomieu*	*Moxostoma anisurum*
20	22	13	0.061	5.2	1.000	***0.000***	*Micropterus dolomieu*	*Moxostoma duquesnii*
20	44	15	0.122	10.4	0.996	***0.016***	*Micropterus dolomieu*	*Moxostoma erythrurum*
20	26	13	0.072	6.1	1.000	***0.000***	*Micropterus dolomieu*	*Moxostoma macrolepidotum*
20	7	7	0.019	1.6	1.000	***0.000***	*Micropterus dolomieu*	*Moxostoma valenciennesi*
20	21	10	0.058	4.9	0.999	***0.005***	*Micropterus dolomieu*	*Notropis buchanani*
20	20	9	0.055	4.7	0.997	***0.014***	*Micropterus dolomieu*	*Noturus flavus*
20	12	0	0.033	2.8	***0.031***	1.000	*Micropterus dolomieu*	*Pimephales notatus*
3	1	1	0	0	1.000	***0.035***	*Micropterus salmoides*	*Pimephales promelas*
3	10	2	0.004	0.4	0.999	***0.035***	*Micropterus salmoides*	*Semotilus atromaculatus*
38	22	19	0.116	9.8	1.000	***0.000***	*Moxostoma anisurum*	*Moxostoma duquesnii*
38	44	27	0.231	19.7	1.000	***0.001***	*Moxostoma anisurum*	*Moxostoma erythrurum*
38	26	20	0.137	11.6	1.000	***0.000***	*Moxostoma anisurum*	*Moxostoma macrolepidotum*

38	7	7	0.037	3.1	1.000	***0.003***	*Moxostoma anisurum*	*Moxostoma valenciennesi*
38	21	17	0.11	9.4	1.000	***0.000***	*Moxostoma anisurum*	*Notropis buchanani*
38	5	5	0.026	2.2	1.000	***0.015***	*Moxostoma anisurum*	*Notropis rubellus*
38	20	13	0.105	8.9	0.991	***0.034***	*Moxostoma anisurum*	*Noturus flavus*
38	7	6	0.037	3.1	0.997	***0.029***	*Moxostoma anisurum*	*Percina caprodes*
3	26	3	0.011	0.9	1.000	***0.026***	*Moxostoma carinatum*	*Moxostoma macrolepidotum*
3	7	3	0.003	0.2	1.000	***0.000***	*Moxostoma carinatum*	*Percina caprodes*
22	44	20	0.134	11.4	1.000	***0.000***	*Moxostoma duquesnii*	*Moxostoma erythrurum*
22	26	15	0.079	6.7	1.000	***0.000***	*Moxostoma duquesnii*	*Moxostoma macrolepidotum*
22	7	7	0.021	1.8	1.000	***0.000***	*Moxostoma duquesnii*	*Moxostoma valenciennesi*
22	21	11	0.064	5.4	1.000	***0.002***	*Moxostoma duquesnii*	*Notropis buchanani*
22	3	3	0.009	0.8	1.000	***0.016***	*Moxostoma duquesnii*	*Notropis photogenis*
22	5	4	0.015	1.3	0.999	***0.015***	*Moxostoma duquesnii*	*Notropis rubellus*
22	20	11	0.061	5.2	1.000	***0.001***	*Moxostoma duquesnii*	*Noturus flavus*
22	12	0	0.037	3.1	***0.020***	1.000	*Moxostoma duquesnii*	*Pimephales notatus*
44	26	22	0.158	13.5	1.000	***0.000***	*Moxostoma erythrurum*	*Moxostoma macrolepidotum*
44	7	7	0.043	3.6	1.000	***0.008***	*Moxostoma erythrurum*	*Moxostoma valenciennesi*
44	7	7	0.043	3.6	1.000	***0.008***	*Moxostoma erythrurum*	*Percina caprodes*
44	10	2	0.061	5.2	***0.034***	0.995	*Moxostoma erythrurum*	*Semotilus atromaculatus*
26	7	7	0.025	2.1	1.000	***0.000***	*Moxostoma macrolepidotum*	*Moxostoma valenciennesi*
26	21	12	0.076	6.4	0.999	***0.003***	*Moxostoma macrolepidotum*	*Notropis buchanani*
26	5	4	0.018	1.5	0.998	***0.029***	*Moxostoma macrolepidotum*	*Notropis rubellus*
26	20	10	0.072	6.1	0.991	***0.033***	*Moxostoma macrolepidotum*	*Noturus flavus*
26	7	7	0.025	2.1	1.000	***0.000***	*Moxostoma macrolepidotum*	*Percina caprodes*
7	2	2	0.002	0.2	1.000	***0.006***	*Moxostoma valenciennesi*	*Notropis atherinoides*
7	21	5	0.02	1.7	0.999	***0.009***	*Moxostoma valenciennesi*	*Notropis buchanani*
7	3	2	0.003	0.2	1.000	***0.017***	*Moxostoma valenciennesi*	*Notropis photogenis*
7	20	5	0.019	1.6	0.999	***0.007***	*Moxostoma valenciennesi*	*Noturus flavus*
6	4	3	0.003	0.3	1.000	***0.001***	*Nocomis biguttatus*	*Pomoxis annularis*
6	10	4	0.008	0.7	1.000	***0.001***	*Nocomis biguttatus*	*Semotilus atromaculatus*
2	2	1	0.001	0	1.000	***0.047***	*Nocomis micropogon*	*Notropis atherinoides*
2	5	2	0.001	0.1	1.000	***0.003***	*Notropis atherinoides*	*Notropis rubellus*
21	5	5	0.015	1.2	1.000	***0.001***	*Notropis buchanani*	*Notropis rubellus*
21	7	5	0.02	1.7	0.999	***0.009***	*Notropis buchanani*	*Percina caprodes*
3	20	3	0.008	0.7	1.000	***0.012***	*Notropis photogenis*	*Noturus flavus*
2	7	2	0.002	0.2	1.000	***0.006***	*Notropis volucellus*	*Percina caprodes*
8	5	3	0.006	0.5	1.000	***0.005***	*Noturus stigmosus*	*Sander vitreus*
12	10	4	0.017	1.4	0.996	***0.031***	*Pimephales notatus*	*Semotilus atromaculatus*
4	10	3	0.006	0.5	1.000	***0.005***	*Poxomis annularis*	*Semotilus atromaculatus*

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Chapter 4

GENERAL CONCLUSION

Conservation efforts are substantially more effective when applied early before irreversible damage occurs to species and their ecosystems. First and foremost, gathering spatial distribution information for the target species (either at-risk species or invasive species) is required for delimiting critical areas that need to be carefully monitored and perhaps protected. Conventional methods are less prone to false positive detections since physical captures of individuals confirm their presence. However, conventional methods have decreased detection sensitivity when attempting to capture rare, cryptic, or inhabit difficult to sample areas. Increasingly, eDNA methods are proving to perform equal to or better than conventional methods (Jerde et al. 2011; Dejean et al. 2012; Mahon et al. 2013; Takahara et al. 2013). Furthermore, conventional methods can unintentionally cause harm to captured individuals, indirect harm to co-occurring species in the same habitat, or damage to the habitat (Santas 2013; Mächler et al. 2014; Evans et al. 2016). Here, I discuss findings from my thesis, which reports on the dynamics of eDNA in flowing freshwater ecosystems and contributes to our knowledge of its efficacy in detecting rare (recently removed and at-risk species), invasive species, and whole fish communities in two watersheds in southern Ontario.

eDNA in lentic systems (e.g. lakes and ponds) is not subject to downstream movement, resulting in lower degradation rates and lower dilution effects with overall greater eDNA retention (Rees et al. 2014). This is not the case in flowing systems where eDNA migration leads to greater dilution effects, physical and chemical degradation, and detection of eDNA in locations where the target species may not occur (Goldberg et al. 2015). In Chapter 2, I aimed to determine the detection sensitivity of residual eDNA in a river after the eDNA source was removed using qRT-PCR analysis. My major finding was that residual eDNA signals were

strongest (lowest mean Ct values) at the source site in 2014 when I began sampling from the source site (2 hours after eDNA source removal) and moved downstream and, in 2015, when I began sampling farther downstream and moved upstream towards the source site (11.5 hours after eDNA source removal). I also found that sampling starting at the source site and moving downstream, resulted in more downstream sites with stronger eDNA signals (up to 960 m downstream). This was due to less time having passed by since I began sampling at the source site allowing for more samples to have higher eDNA concentrations, whereas in 2015 more time passed by during sampling when approaching the source site, resulting in a stronger residual eDNA detection only at the source site. In Chapter 3, I used eDNA analysis to detect four target species and other non-target species in Grand River and Sydenham River and compared my results with past capture records collected by DFO in selected sites. Overall, the majority of positive eDNA detections were in sites adjacent to sites that had individuals captured using conventional methods (Table 3.3). Many reasons exist for why eDNA detections do not always occur at the same site as captured individuals, and they include movement of individuals, resulting in eDNA production in several sites and the migration and degradation of eDNA in flowing systems. However, my work has shown that spatial and temporal sampling can overcome some of the challenges of working in flowing systems.

Several studies have used eDNA signal strength to infer species abundance or biomass (Takahara et al. 2012; Pilliod et al. 2013; Eichmiller et al. 2014). Greater species abundance or biomass in an area will contribute to higher eDNA production in the area and thus, would produce stronger eDNA signals. In Chapter 2, the strongest residual eDNA signals were at the source site where Atlantic Salmon water was released – contributing to a greater number of eDNA molecules at the source site. This is despite the source having been removed a couple hours earlier. Generally, studies examining eDNA signal strength relative to species abundance

and biomass focus on single species, similar to what I did in Chapter 2. Although eDNA quantification was beyond the scope of Chapter 3, it is interesting to note that sites that had a low number of captured individuals did not always produce a low number of eDNA sequence reads, nor did sites with a high number of captured individuals necessarily produce a high number of eDNA sequence reads (Table 3.3). As explained above, this is likely due to the movement of individuals among sites and the dilution of eDNA in flowing systems, which increases the variation of eDNA concentration and degradation rates across sites. Also, this variation may be attributable to the semi-quantitative nature of NGS, whereby, it is reasonable to assume that highly abundant species will contribute to a greater sequence read number but DNA concentrations (and thus, proper quantification) will differ based on eDNA extraction methods and primer bias (Pompanon et al. 2012; Renshaw et al. 2015).

eDNA analysis is an innovative tool with important conservation management applications. I demonstrate in Chapter 2 that residual eDNA signals can be used to pinpoint eDNA sources for a single target species, even after the eDNA source was removed from a flowing system. Furthermore, in Chapter 3, I was able to detect at-risk species, invasive species, and common species in two large Great Lakes tributaries by using a hybrid primer approach together with NGS. I aimed to detect 67 fishes (including my four target species) that were captured in previous years by conventional methods, and was able to detect 51 species (76.1%) which includes my four target species. This was after one sampling season and use of a limited number of river samples and, if more samples were re-analyzed with the use of different gene markers, it is possible that more species would have been detected. Another important aspect of the exceptionally high detection sensitivity of eDNA coupled with PCR-NGS is that I was able to detect rare species, including my three target at-risk species. This detection was despite the vast majority (77.0%) of NGS sequence reads matching the highly invasive and very abundant

Round Goby. Hence, eDNA offers the opportunity to characterize species communities even in systems dominated by one or a few common/invasive species that contribute to the majority of the eDNA in the system. Another advantage of eDNA analysis is that the filters may be stored for future use to be re-analyzed using different genetic markers to assess different species communities or to strengthen the presence/absence data gathered.

Future Directions

The main goal of this thesis was to assess the robustness of eDNA analysis in characterizing flowing aquatic ecosystems. It is important that future studies continue to optimize eDNA as a tool in a variety of systems to expand our knowledge of the relationship between eDNA detection and target species presence/absence. Although presence data are important for species conservation and management, the disadvantage to this approach is that eDNA undergoes degradation and dilution (displacement in flowing environments), making it vulnerable to false negatives. This is problematic if eDNA methods are applied in systems that lack capture-based data to confirm species composition and thus, difficult to identify possible false negatives or positives. Quantitative platforms improve our confidence of species location by measuring the strength of eDNA detection signals. It is recommended that future studies determining eDNA dynamics in flowing ecosystems should include additional variables such as flow (volume and direction) at each sample point and use systems that differ in environmental variables (e.g. pH, turbidity, temperature) to determine how environmental variation can affect residual eDNA persistence. Measuring different variables will help in standardizing the eDNA method, and how to optimize the eDNA extraction process for a variety of habitats (see Appendix A). Additionally, quantifying the number of individuals using eDNA samples can be difficult; however, several studies have found that greater species abundance and biomass

resulted in stronger eDNA signals in controlled experiments. Determining species abundance without the use of potentially harmful conventional methods is also more important for at-risk species. Therefore, I propose that future studies should include several sample time points (e.g. different seasons) per sample site, as sites with high species abundance (e.g. favourable habitats) would more often produce strong eDNA signals due to increased eDNA production in these areas, and sites with fewer individuals would have weaker or more variable (i.e. inconsistent eDNA production rates) eDNA signals. Although this does not identify a precise census number, this approach may be useful when using semi-quantitative NGS platforms or qRT-PCR analysis for a more quantitative/qualitative measure (e.g. site A consistently produces stronger eDNA signals or greater sequence reads for a target species and is likely to have more individuals at that site).

Likewise, the use of multiple molecular markers targeting short amplicons (ideal for highly degraded eDNA and NGS platforms) and the incorporation of generic primers (targeting the known community or known co-occurring species) can be useful for rapid assessment of community composition and the detection of species at risk or invasive species as seen in Chapter 3. I recommend that targeted molecular markers be designed to be highly specific for a single species, to avoid possible mispriming in eDNA samples. I also recommend that, if the target species has a well-known breeding season, eDNA sampling should be conducted during these times for greater eDNA detection, as eDNA production rates are found to be greater and more likely to be sampled effectively (Fukumoto et al. 2015; Spear et al. 2015). Moreover, I suggest that the use of eDNA analysis, especially in large lotic systems, be followed by targeted conventional sampling for increased species detection and confirmation of the eDNA results. If the eDNA analysis could not detect common species known to be abundant in the system, it calls into question the validity of eDNA results concerning rare species (as seen with Chapter 3

and the use of more targeted species primers, owing to improper amplicon target size; see Appendix A for caveats on primer design).

Overall, eDNA methods would appear to be a useful, complementary tool alongside conventional methods. First, it can be used to pinpoint areas that are likely to contain target species based on eDNA concentrations measured using qRT-PCR. Second, although eDNA analysis is not immune to false negative and false positives, it's higher detection sensitivity, lower costs, and logistic difficulties allow it to be used initially to characterize large systems for a quick assessment of community distribution (Yamamoto et al. 2016). The combination of eDNA and capture validation has also been recommended in recent eDNA studies (Tréguier et al. 2014; Wilson et al. 2014; Fukumoto et al. 2015; Rees et al. 2015). Finally, eDNA applications are not limited to aquatic species spatial delineation. It has been used to monitor the spread of disease in aquatic environments (Walker et al. 2007), mammals (Dalén et al. 2007; Zhu et al. 2011; Nichols et al. 2012), plants (Schnell et al. 2010), and insects (Schnell et al. 2010). This demonstrates the broad applicability of eDNA for various environments and spatial scales, making it a necessary and innovative tool for global conservation.

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APPENDIX A
TROUBLESHOOTING EDNA SAMPLES AND PRIMER DESIGN

The extraction of DNA from environmental samples will include the extraction several PCR inhibitors such as fulvic acids or humic acids. The eDNA extraction process continues to improve with the implementation of more robust protocols and DNA extraction kits, however these kits or protocols are often expensive. Throughout my research I have come across techniques that can help optimize the extraction process and amplification of eDNA. I have included my tips as a list below.

1. There are kits designed specifically for eDNA samples (i.e. MOBIO Power Soil/Water® DNA Isolation kits), however, if they cannot be used due to pricing or availability, phenol-chloroform is preferred as it removes proteins, salts, and inhibitors.
2. The use of glass beads (I used 1.0 mm glass beads from Fisher Scientific LTD, BioSpec. Cat. No. 11079110) and homogenization of filters is required to fully digest the filters and release eDNA from cells. Instead of extracting ½ filter as I did, break the sample into two and extract them as ¼ filters which reduces the concentration of inhibitors. Samples can then be combined into a single tube at the DNA elution step.
3. Research suggests to include an incubation stage when CTAB digestion buffer is added to the samples for approximately 15-30 minutes at 55-60 ^{o}C (before PCI addition and homogenization). It is also suggested to include polyvinylpyrrolidone (PVP) and β-mercaptoethanol if samples are taken from systems containing abundant plant matter. These two ingredients help remove phenolic compounds (also PCR inhibitors) released by plants.
4. At the isopropanol and sodium acetate stage, let samples sit overnight at -20 ^{o}C for increased DNA precipitation. I suggest to use two washes with ice cold 70% ethanol

instead of one wash (as I did). Some DNA pellets may still look brown at this point (indicates they will be inhibited from my experience).

5. If DNA pellets are still brown, try to elute in 60+ µL of TE buffer. I used 30 µL which likely concentrated PCR inhibitors. When eluting, include 1-2 µL of RNase A (20 $mg \cdot mL^{-1}$) to remove contaminating RNA. Once added and DNA eluted, centrifuge for a few seconds then incubate for at least an hour at 37 ^{o}C to properly dissolve the DNA pellet. Do not vortex or use a pipette to mix the sample at this stage as this will shear the genomic DNA. Store the DNA at -20 ^{o}C.
6. My next suggestion is to keep an DNA stock that will remain in the freezer to avoid repeated freezing and thawing of the DNA samples, as this likely contributed to increased DNA degradation. For example, if DNA is eluted in 60 µL, put approximately 50 µL in a plate and keep the remaining 10 µL in a separate plate that you can be used more often. This keeps the majority of extracted DNA in a stable form.
7. Finally, diluting the samples further in TE or ddH_2O prior to PCR helps by diluting PCR inhibitors and allowing PCR reagents to work more efficiently. I found that a 10-fold dilution (i.e. 2 µL DNA and 18 µL ddH_2O) helped in amplification.
8. **Primers:** If targeting one specific species, use Primer BLAST and at least a second program of any choice (i.e. Net Primer or Primer 3) to design species-specific primers. Using multiple programs helps to confirm the specificity of the primer. Ascertain that the forward and reverse primer have similar melting temperatures (target annealing temperature should be between 55 ^{o}C – 65 ^{o}C).
9. Length of primer should be 18-25 bp in length and for eDNA samples, target fragment size (including primers) should be 100-250 bp (ideal for degraded DNA and NGS).

10. Use Primer Map to quickly map degenerate primers using sequences of your target species, since Primer BLAST does not work with degenerate primers. This is simply to ensure the degenerate primers can be mapped to your target species without having to replace the degenerate bases with a known base and testing every possible combination.
11. For any primer, avoid degenerate bases within 5 bp of the 3' end for optimal annealing. If a degenerate base is required near the 3' end, I suggest ordering the primers with each type of nucleotide to avoid ordering primers with degenerate bases near the 3' end. This will result in more primers to use, but will avoid potential mispriming.
12. If designing multiple primers to be used in the same reaction, ensure they all target the same fragment size and have similar melting temperature.
13. To test primer effectiveness, I suggest not testing it on tissue samples alone because tissue DNA concentrations are much higher than natural eDNA concentrations. Instead, take water samples from systems where you have the target species, or try to spike the water samples and extract in the same manner. This will also help to test if your extraction process is effectively removing inhibitors and if your PCR amplification is optimized.

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